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MOLECULAR MECHANISMS OF ANTIBODY DIVERSIFICATION IN HUMAN B-CELLS

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Molecular mechanisms of antibody diversification in human B-cells

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To my family

ABSTRACT

B-cells undergo several somatic rearrangement/mutational processes during development to diversify their immunoglobulin (Ig) receptors. First, the antigen binding Ig variable (V) region is assembled through V(D)J recombination. Later, B-cells undergo class switch recombination (CSR), which exchanges the constant region and thus Ig isotype and immune effector function. In addition, point mutations are introduced into the Ig V regions, to increase the affinity between the Ig and antigen, by somatic hypermutation (SHM). These processes rely on numerous DNA damage response (DDR) and DNA repair proteins, such as those from the classical non-homologous end-joining (c-NHEJ) pathway. The aim of this thesis was to elucidate the functions of some of these proteins during antibody diversification in human cells.

In ***Paper I***, cells from patients with Cornelia de Lange syndrome (CdLS), with mutations in the gene encoding the cohesin loader NIPBL, were studied. The analysis of CSR junctions from these patients revealed an altered repair pattern. It was characterized by reduced direct end-joining and increased microhomology (MH) usage, indicating a shift from c-NHEJ to alternative end-joining (A-EJ). A role for NIPBL in promoting NHEJ was shown to be conserved in yeast. Furthermore, the early recruitment of the DDR factor 53BP1 to DSBs was impaired in the NIPBL-deficient cells, suggesting a mechanism for the involvement of NIPBL/cohesin in NHEJ.

In ***Paper II***, the Ig heavy (H) chain and T cell receptor (TCR) β chain V region repertoires were analyzed in CdLS patients by high through-put sequencing methods. The cells from the CdLS patients showed reduced lymphocyte receptor diversity and a skewed V gene usage, which seemed dependent on the location of the genes at the IgH and TCRβ loci. Furthermore, the frequency of SHMs in the IgH V regions was reduced. These results might imply a role for cohesin in the regulation of SHM, as well as in processes ensuring that V genes throughout the Ig locus are used during V(D)J recombination.

In ***Paper III***, the CSR junctions were analyzed in individuals deficient in the tumor suppressor BRCA1. The CSR junctions showed several aberrations, including increased MH usage, elevated frequency of intra-switch deletions and unusual insertions containing inversions. Alterations were also observed at the CSR-junctions from patients with defects in the BRCA1-associated proteins BRIP1, BRCA2, CtIP and RNF168. Thus, it seems as BRCA1 together with its interaction partners play an important role in promoting the c-NHEJ pathway during CSR.

In ***Paper IV***, CSR junctions were examined in the two only patients described with DNA-PKcs-deficiency to date. Furthermore, CSR-junctions from DNA-PKcs-deficient mice and the Ramos B-cell line, transfected with a switch plasmid and treated with DNA-PKcs inhibitor, were studied. The role of DNA-PKcs in CSR has been unclear, but the analysis of CSR junctions from all DNA-PKcs-deficient models revealed a shift to A-EJ, suggesting that CSR, indeed, is affected in DNA-PKcs-deficient cells.

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J. Exp. Med., 2013, 18;210(12):2503-13

II. **Björkman A**, van der Burg M., Kipling D. K., Walters D. D., Pan-Hammarström Q.
Altered usage of 3' proximal immunoglobulin variable genes in patients with the cohesinopathy Cornelia de Lange syndrome
Manuscript

III. **Björkman A**, Qvist P, Du L, Bartish M, Zaravinos A, Georgiou K, Børglum AD, Gatti RA, Törngren T, Pan-Hammarström Q.
Aberrant recombination and repair during immunoglobulin class switch recombination in BRCA1-deficient human B-cells
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Submitted to J. Allergy. Clin. Immunol.

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LIST OF ABBREVIATIONS

53BP1	Tp53 binding protein 1
A	Adenine
A-EJ	Alternative end-joining
AID	Activation induced cytidine deaminase
AgR	Antigen receptor
A-T	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia mutated
BCR	B cell receptor
BER	Base excision repair
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BRIP1	BRCA1 interacting protein 1
C	Cytosine
CD	Cluster of differentiation
CdLS	Cornelia de Lange syndrome
CSR	Class switch recombination
CTCF	CCCTC binding factor
CVID	Common variable immunodeficiency
D gene	Diversity gene
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	DNA double strand break
FA	Fanconi Anemia
G	Guanine
H	Heavy chain
HR	Homologous recombination
Ig	Immunoglobulin
ISD	Intra-switch deletion
J gene	Joining gene
MH	Microhomology
MLH1	MutL homologue 1

MMR	Mismatch repair
MSH2	MutS homologue 2
NHEJ	Nonhomologus end-joining
NHL	Non-Hodgkin lymphoma
NIPBL	Nipped-B like protein
NK cell	Natural killer cell
PALB2	Partner and localizer of BRCA2
PARP1	Poly(ADP-ribose)polymerase 1
PAXX	Paralogue of XRCC4 and XLF
PID	Primary immunodeficiency disorder
PMS2	Postmeiotic segregation increased 2
RAD21	Radiation-sensitive mutant 21
RAG	Recombination activation gene
RIF1	Rap1-Interacting Factor 1 Homologue
RNF	Ring finger protein
SCID	Severe combined immunodeficiency
SHM	Somatic hypermutation
S region	Switch region
SMC1A	Structural maintenance of chromosomes 1A
SMC3	Structural maintenance of chromosomes 3
SSB	Single strand break
T	Thymine
TCR	T cell receptor
U	Uracil
UNG	uracil-DNA glycosylase
V gene	Variable gene
XLF	XRCC4-like factor
XRCC4	X-ray repair cross-complementing protein 4

1 INTRODUCTION

1.1 THE ADAPTIVE IMMUNE SYSTEM

Most multicellular organisms, ranging from plants and insects to birds and mammals, have developed immune systems to protect themselves from outside invaders.[1] It requires the ability to distinguish foreign molecules, such as pathogenic agents (pathogens) including viruses, bacteria, fungi and parasites, from the host organism's own components and the capacity to eliminate the pathogens.

The first type of defense that developed during evolution is the innate immune system.[1] It acts immediately upon an infection and recognizes structures that are shared among groups of pathogens. It includes anatomical barriers, such as the skin and mucosal surfaces, as well as pattern recognition receptors that bind motifs that are present on infectious organisms and signals to the immune system.[2] Furthermore, specialized immune cells, including phagocytes, which can ingest pathogens and natural killer cells, which kill infected cells, belong to the innate immune system.[1]

A second immune branch started to develop in jawless fishes, the adaptive immune system, which exists in vertebrates.[3] The adaptive immune system requires longer time to react compared to the innate immune system, but can develop recognition of specific infectious microorganisms. Furthermore, this specificity can be stored in memory cells that can quickly start an immune response if the host encounters the same pathogen later in life. The adaptive immune system consists of two cell lineages: B and T lymphocytes.

Nevertheless, the innate and adaptive immune branches are dependent on each other and cross talk occurs between them. A component of the innate immune system, which plays a role in connecting the two systems, is the dendritic cell. Dendritic cells present antigens, parts of (mostly) foreign molecules, to lymphocytes and can activate the adaptive immune response.[4] Furthermore, immune cells with characteristics of both branches have been discovered, such as innate lymphoid cells, which are lymphocytes without a particular antigen specificity[5] and invariant NKT cells, resembling both NK and T cells.[6]

1.1.1 B lymphocytes

The B cell was named after the avian organ bursa of Fabricius, in which it was first discovered.[7] The letter B also coincides with its developmental origin in mammals, the bone marrow. However, long before the B cell was revealed, antibodies or immunoglobulins (Igs), which are produced by B-cells, were described. In 1890, Emil von Behring and Shibasaburo Kitasato termed them antitoxins, since they could neutralize the toxins of diphtheria and

tetanus.[8] Later, the components of Igs were elucidated and it was found that Igs were composed of three fragments. One of these, the Fc (crystallizable fragment) or constant (C) region, conferred the Igs immune effector functions, whereas the other two were antigen-binding (Fabs) and contained the variable (V) region.[9] The Ig V region can bind to various types of antigen structures, including proteins, polysaccharides, lipids and nucleic acids. The Ig composition can also be described as consisting of two identical heavy (H) chains comprised of V and C regions, either μ (IgM), γ (IgG), α (IgA) or ε (IgE), and two identical light (L) chains, κ or λ, which are attached by disulfide bonds (Fig. 1). Igs can either act as receptors, when bound to the B cell membrane, or be secreted from the cells in their soluble form.

The different Ig classes have various biological functions and distributions within the body. IgM and IgD are the first Ig classes expressed, whereas IgG is the most prevalent Ig in serum.[10] In contrast, on mucosal surfaces IgA is most predominant,[11] whereas IgE has the lowest overall abundance and seems to mainly function in the defense against parasites, as well as in mediating allergic reactions.[12] Igs can have neutralizing effects by encircling or binding to toxins or viruses and thereby inhibit their functions.[11] Furthermore, Igs may mark pathogens for targeting of innate immune cells, such as phagocytes, neutrophils and NK cells, as well as activate immune functions, including the complement system.[10]

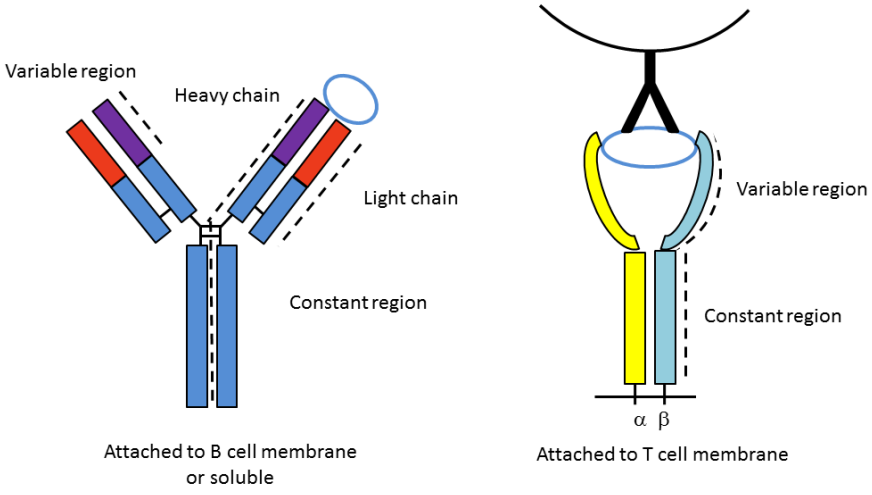


Figure 1: The B and T cell receptors

Schematic picture of immunoglobulin (Ig) is shown to the left. Constant (C) regions are depicted in blue and the variable (V) regions, binding an antigen (blue circle), in red and purple. The Ig consists of two identical heavy chains and two identical light chains. A T-cell receptor composed of αβ chains is shown to the right. Each chain is composed of a C region and a V region and the latter binds to an antigen presented by an MHC-molecule.

1.1.1.1 The clonal selection theory

The discovery of Igs created a new puzzle regarding how the Igs could become so diverse that they could theoretically recognize all pathogens. Some immunologists believed that the antigens themselves served as templates for instructing the B-cells to produce antibodies that

could bind to them (the instructive theories).[13] Others argued that the antigens bound to Igs that already preexisted in the organism (the selective theories). A follower of the latter was Frank Macfarlane Burnet who, in 1957, proposed the clonal selection theory.[14] According to this theory, B-cells with a great variety of antigen specificities already exist in the body and each B cell only confers a single specificity. Furthermore, those cells that carry receptors that do not bind antigens will be eliminated, whereas the cells that carry receptors that bind to antigens will expand. A year later it was also shown experimentally that one B cell only produces one antibody specificity.[15]

1.1.1.2 B cell development

A B cell can go through several developmental and maturation stages and many of them are dependent on somatic DNA rearrangement/mutational processes that will diversify their Ig receptors.[16] These include V(D)J recombination, which assembles the H and L chains, class switch recombination (CSR), which exchanges the Ig isotype, and somatic hypermutation (SHM), which can improve the affinity between the antibody and antigen (Fig. 2).

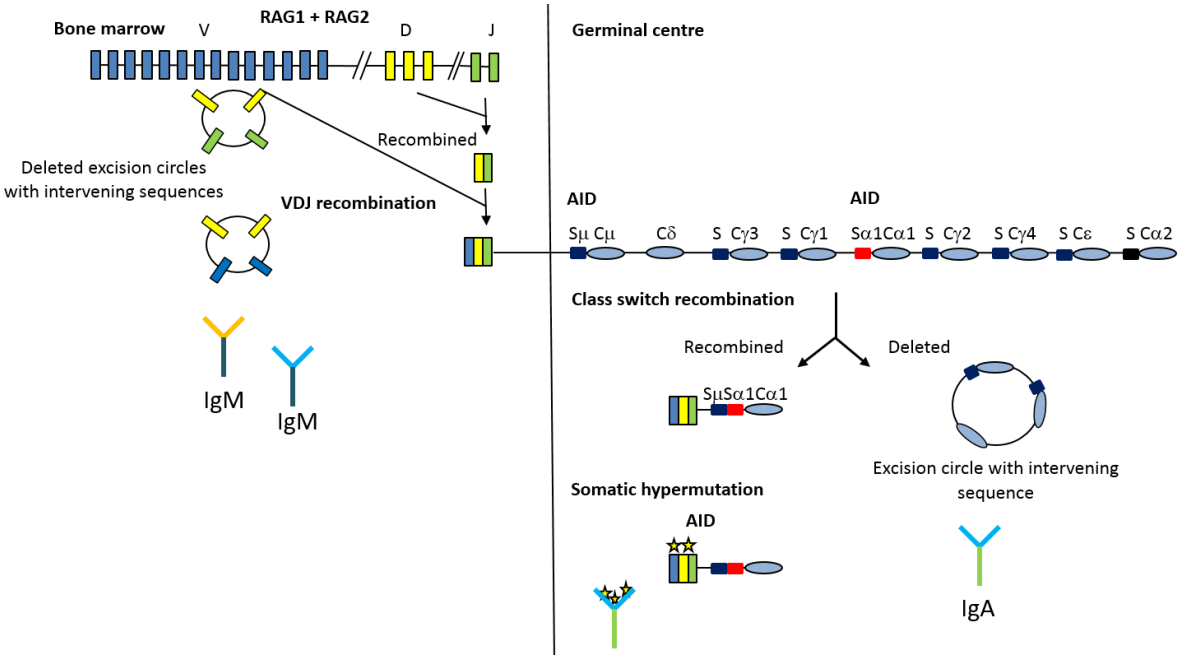


Figure 2. ***The antibody diversification processes V(D)J recombination, class switch recombination and somatic hypermutation.*** V(D)J recombination occurs in the bone marrow and assembles V, D and J genes through a deletion/recombination process initiated by the RAG1 and RAG2 enzymes, into the Ig V region. In the germinal center, CSR is initiated by AID and here exemplified by the exchange of the Cμ region gene with the Cα1 region gene in a recombination/deletion process, resulting in an exchange of IgM to IgA isotype expression. In the germinal center, AID also initiates SHM, which introduces point mutations, marked by starts, in the Ig V region to increase the affinity between the Ig and antibody.

The hematopoietic precursor cells that give rise to B-cells reside in the bone marrow.[17] The developmental process begins at the pro-B cell stage by the assembly of the H chain by J-D, followed by JD-V, rearrangement.[16] The L chain is rearranged through V-J recombination

during the pre-B cell stage and when this process is completed, the B-cell has reached the immature B-cell stage. Subsequently, the B cell is tested against self-antigens and if it binds to self it will not continue the maturation process. Instead, it could undergo receptor-editing, during which the B-cell rearranges its L chain a second time to change its specificity.[18] This check point ensures that only B-cells that are tolerant to the organism's own components, will exit the bone marrow. If the B-cell encounters an antigen that can bind to its receptor, it will become activated and migrate to germinal centers, which are structures in secondary lymphoid organs, such as the spleen, lymph nodes and Peyer's patches in the intestine.[19] There it will proliferate, as well as go through SHM and CSR. The B-cells can thereafter mature into plasma cells, excreting Igs, or memory B-cells, which will reside in the organism and can become activated if they encounter antigens later in life.

1.1.2 T lymphocytes

Although T-cells originate from the bone marrow, they received their names from the organ where most of their development occurs, the thymus.[20] Similarly to B-cells, T-cells express receptors, T-cell receptors (TCRs), which can bind to antigen. However, in contrast to B-cells, the TCRs are always attached to the T-cell membrane. Furthermore, the TCR only binds to a small peptide of the antigen that has been presented to it by a major histocompatibility complex (MHC) molecule.[21] MHC class I molecules are present on nearly all the cells within the organism and are recognized by $CD8^+$ T-cells, which are mostly referred to as cytotoxic T-cells.[20] The organism's cells continuously display peptides from their intracellular cytoplasm. If a foreign antigen, such as from a virus or tumor is exhibited, the cell will be eliminated by the cytotoxic T-cells to prevent further spread of the infection or the malignant cells. MHC class II molecules, on the other hand, are only present on professional antigen presenting cells, such as dendritic cells and B-cells, which present peptides from the extracellular environment. Antigens presented by MHC class II molecules are detected by $CD4^+$ T-cells, which are most often referred to as helper T-cells and can aid the immune response by secretion of cytokines, signaling molecules that activates immune components, such as B-cells.[20]

The TCR is a heterodimeric glycoprotein consisting of α and β chains, in most T-cells, or γ and δ chains, in a subset of cells, connected by disulphide bonds.[20] Similarly to the Igs, the TCR consists of V and C regions (Fig. 1), of which V binds the MHC-antigen complex, whereas the C region is attached to the cell membrane. Unlike Igs, the C region of the TCR cannot be exchanged and its V region will not undergo affinity maturation. Since the TCR binds both to the antigen and the MHC molecule, its V region specificity is directed to both molecules, so called MHC restriction.[20]

1.1.2.1 T-cell development

The hematopoietic stem cells that give rise to T-cells reside in the bone marrow. However, already at the early thymic progenitor stage, these cells migrate to the thymus, where rearrangement begins. Pro T-cells, which do not express CD4 or CD8 (double negative), undergo TCRβ rearrangement through D-J followed by V-DJ recombination. Subsequently, CD4 and CD8 are expressed (double positive) and TCRα V-J rearrangement may begin. When the V region assembly is completed, the TCR can undergo positive and negative selection, which prevents auto-reactive T-cells from exiting the thymus. T-cells having receptors with too high-affinity for the antigen-MHC complex are then eliminated,[21] whereas those that can either bind peptide-MHC class I or II become $CD8^+$ or $CD4^+$ T-cells, respectively. Finally, the cells can exit to the periphery. Both $CD4^+$ and $CD8^+$ T-cells can develop into memory T-cells.

1.2 V(D)J RECOMBINATION

According to the clonal selection theory, B (and T) cells must produce an immense number of different antigen receptors (AgRs). It was long considered an enigma how the B and T-cells could produce V regions with such a variability[13]. In the 1970s, when the V(D)J recombination process was discovered, the knowledge about the genome and the number of genes it contained was limited. Still, it seemed unlikely that our genomes would contain millions of genes only to encode the immune system. This paradox was thus explained when Susumu Tonegawa presented how V(D)J recombination could give rise to millions of antigen specificities from a limited number of genes by assembling different combinations of variable (V), diversity (D) and joining (J) genes into the V regions of Igs[22] (Figure 2). Since then, more details have been elucidated regarding the process, such as how the V, D, and J genes are targeted, excised and recombined.

1.2.1 The accessibility hypothesis

All cells carry the genes encoding the Igs and TCRs, but as these are only rearranged and expressed in lymphocytes, these genes are tightly packed in chromatin in most cells. In lymphocytes, V(D)J recombination must also occur during specific developmental time points and a rearrangement resulting in a productive V region may only occur on one allele. Thus, according to the "accessibility hypothesis", proposed more than thirty years ago, several processes are involved in making the chromatin embedded genes available to the recombination machinery.[23,24]

The theory arose after the observation of germline (sterile) transcription, which does not result in the expression of proteins, during V(D)J recombination.[24,25] The transcription started

at the V gene's promoter and ended at the recombination site. It has become clear that germline transcription is necessary for V(D)J recombination, since the inhibition of transcription at the J gene cluster at the TCRα locus, completely blocked the recombination of the J segments downstream of the inhibition site.[26]

Another form of transcription, antisense transcription, which occurs in the opposite direction and spans several V genes, is also observed during V(D)J recombination.[27] Apart from making the locus accessible, antisense transcription seems to facilitate locus contraction. Then several DNA-loops are created, forming a rosette-like structure of the AgR locus[28]. Some AgR loci span several 100 kbs and locus contraction ensures that distally located V genes get an equal chance for recombination as the proximal V genes.

In addition, an open chromatin structure is created by histone modifications, such as hypermethylation of lysine4 at histone (H) 4 and acetylation of H3/H4 during V(D)J recombination.[29] Moreover, the repositioning of the AgR locus within the nucleus appears to be of importance. Accordingly, the IgH and Igκ loci have been shown to be located in the peripheral parts of the nucleus, with a repressive chromatin environment, in hemapoietic progenitors and pro T-cells, but in a central location in pro-B-cells.[30]

1.2.2 RAG-mediated DNA double strand breaks

When the Ig or TCR locus has become accessible, the lymphocyte specific recombination activation gene (RAG) 1[31] and RAG2[32] can bind to the locus and initiate the V(D)J recombination process. The RAG enzymes bind to specific recombination signal sequences (RSS) at the borders of the V-, D- or J-genes that will recombine. The RSS consists of conserved AT-rich nonamer and palindromic heptamer motifs, which are adjacent to non-conserved 12 or 23 bp spacer sequences[33,34] (Fig. 3). According to the 12/23 rule, only spacers of different size can be paired, which guarantees that merely V-D-J (at IgH and TCRβ, δ) or V-J (IgL, TCRα, γ) recombination occurs.

The RAG complex initiates the DNA double strand break (DSB) formation process by inserting a single stranded (ss) nick at the coding strand that creates a free hydroxyl group at the 5'end (Fig. 3). The hydroxyl group will subsequently attack the other strand, through a trans-esterification reaction, and form a covalently bound hairpin loop at the coding ends, which resides in the chromosome. The deleted, extrachromosomal sequence instead contains blunt, 5'phosphorylated signal ends.[33]

1.2.3 Repair of DNA double strand breaks

DSBs are one of the most severe DNA lesions and if unrepaired they can result in cell death, whereas if misrepaired they could cause genomic rearrangements, translocations. DSBs can be generated from external and internal stresses to the cell, such as ionizing radiation, free

radicals and replication fork collapses.[35] Moreover, DSBs can be intermediates of physiological processes, including V(D)J recombination and CSR. There are two major repair pathways of DSBs, non-homologous end-joining (NHEJ) and homologous recombination (HR).[35]

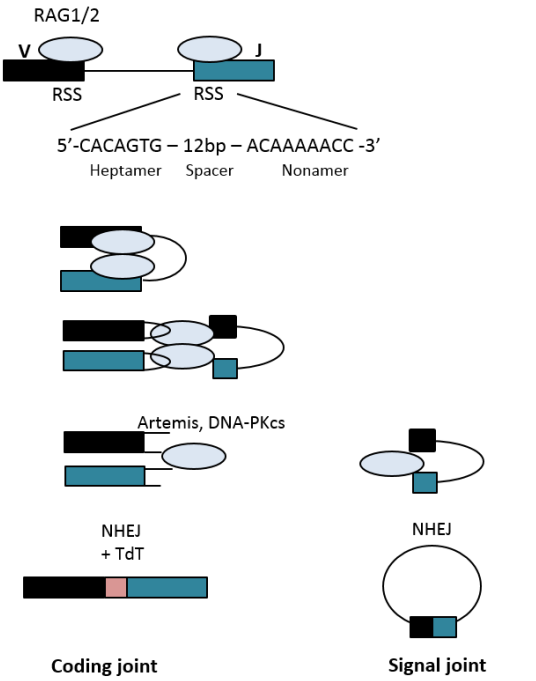


Figure 3. ***Formation of coding and signal joints during V(D)J recombination*** The RAG1/2 complex binds at RSS sequences at the genes that will recombine and locates them in close proximity in the paired complex. Subsequently, the RAG enzymes create hairpin loops at the chromosomal coding ends, whereas blunt signal ends in the deleted sequence. These are then repaired by components from the NHEJ pathway. Additional diversity is created at the coding joint through the deletion or insertion of nucleotides.

In brief, HR is initiated by DNA end resection, which creates 3' ssDNA, followed by strand invasion into undamaged, homologous DNA, usually the sister chromatid.[36] DNA can subsequently be copied from the homologous sequence and the newly synthesized strand will then dissociate and the DNA ends will be re-ligated. HR relies on numerous proteins, such as BRCA1, BRCA2, CtIP and RAD51.[36] Furthermore, HR is restricted to the S/G2 phases when two chromatids are available and it is considered error-free, since any missing information can be copied from the other chromatid.

NHEJ, on the other hand, can act throughout the cell cycle, since it joins the two DNA ends directly, or with a few base pairs overlap, so called microhomologies (MHs).[37] It is seen as error-prone as nucleotides can be lost or inserted during the event. The classical NHEJ (c-NHEJ) factors include Ku70, Ku80, DNA-PKcs, Artemis, DNA ligase IV (Lig4), XRCC4 and Cernunnos (XLF).[35] When a c-NHEJ factor is missing, alternative end-joining (A-EJ) pathways may act and these often requires longer stretches of MHs to join the broken ends (see section 1-3-4). More details about the involvement of c-NHEJ in V(D)J recombination and CSR can also be found in sections 1.2.4 and 1.3.3, respectively.

1.2.4 Repair of coding and signal joints

Since RAG2 is only expressed during the G1 cell cycle phase, the RAG-induced DSBs are repaired by the c-NHEJ pathway.[38] Moreover, the RAG enzymes seem to direct the repair to the NHEJ pathway.[39,40] The inherent low-fidelity in the repair process is also suitable for creation of additional diversity at the coding junctions.

As the signal ends are blunt, whereas the coding ends are shielded in the hairpin, the resolution and recombination of these structures rely on slightly different sets of DNA repair factors. The opening of the hairpin requires the Artemis nuclease,[41] which depends on DNA-PKcs for its activation. If the hairpin is opened asymmetrically, it will either result in deletions or additions of a few (usually 1-2) palindromic (P) nucelotides. Furthermore, the lymphoid-specific enzyme, terminal deoxynucleotidyl transferase (TdT), can add non-template (N) nucleotides to the junctions. These alterations further add to the diversity of the VDJ junctions, so called junctional diversity.[34]

The repair of coding and signal joints is dependent on the Ku70/80 heterodimer, which seems to be the first proteins that bind to the DNA ends.[34] The DNA ends are subsequently ligated by the XRCC4/Lig4/XLF complex. The coding ends also activate factors from the DNA damage response (DDR), including Ataxia-telangiectasia mutated (ATM), which stabilizes the post cleavage complex.[42] However, many other DDR factors, such as 53BP1 and H2AX seem only to have modest or no impact on the repair during V(D)J recombination.[43]

Several DDR and NHEJ factors have not been thought to be required for the repair of coding and/or signal ends. However, studies on double knock out mouse models have shown that their full contributions to the process have been masked by their overlapping functions. Consequently, severe V(D)J recombination defects have been revealed in the combined deficiency of ATM and DNA-PKcs, which could be due to that they share common substrates.[44,45] Furthermore, although XLF is required for V(D)J recombination in human cells,[46] mouse cells seems to be less dependent on XLF during V(D)J recombination.[47] However, redundant functions between XLF and DNA-PKcs[48] and between XLF and the DDR factors 53BP1,[49] H2AX or ATM[50] have been revealed. Thus, mice with the combined deficiency in XLF or any of the mentioned proteins have striking V(D)J recombination defects.

1.3 CLASS SWITCH RECOMBINATION

CSR involves the formation of DSBs in switch (S) regions, intronic sequences located upstream of each C region gene. The subsequent synapsis and recombination of the Sμ with a downstream S region (Sγ, Sα or Sε), result in an exchange of Ig class expression from IgM to IgG, IgA or IgE[51,52] (Fig. 2). The analysis of the repair pattern at the recombined S regions,

CSR junctions, may be used to deduce the DNA repair pathways used in CSR.[51] A shift from the direct end-joining to the use of longer MHs often indicates a defect in c-NHEJ.

1.3.1 AID-initiated creation of DNA double strand breaks in switch regions

CSR, similar to V(D)J recombination, requires germline transcription.[53] The Cμ promoter is constitutively active, whereas the downstream C region promoters are induced by specific cytokines.[54] The C region genes are structured as transcriptional units, starting with a promoter, followed by an intervening exon, an intronic S region and ending with the C region gene.[54] The S regions are guanine (G) rich, repetitive sequences essential for CSR. These regions increase the occurrence of R-loop formations, DNA:RNA hybrid structures that facilitates ssDNA creation and correlates with CSR efficiency.[55,56] The length of the S regions is of relevance for CSR and the longer the S region, the higher is the efficiency of switching.[55,57] One of the main functions of germline transcription seems to be to make the S regions accessible for activation induced cytidine deaminase (AID), which only binds to ssDNA.[58] AID appears to be recruited to transcribed genes through its interaction with Spt5, a protein associated with stalled RNA polymerase II.[59] Furthermore, the S regions contain numerous RGYW/WRCY (R=purine, Y=pyrimidine, W=A/T) motifs, which contain the AID binding hotspots WRC/RGY. However, in contrast to the RAG enzymes, AID does not specifically target conserved sequences, but can bind throughout the S regions.

AID is a B-cell specific molecule and member of the APOBEC family of cytidine deaminases.[53] It initiates the CSR process by deaminating cytosines (C) into uracils (U) in the S regions.[60] AID was discovered more than 15 years ago when its expression was shown to be upregulated 10-fold after cytokine stimulation for CSR.[61] Deficiency in AID results in a complete abolishment in CSR, as well in SHM (see section 1.4) in mice[60] and in humans.[62]

The processing of AID-induced Us by proteins from the base excision repair (BER) and mismatch repair (MMR) pathways may result in DSB formation (Fig. 4).[51] Accordingly, mouse cells double deficient in the BER protein uracil-DNA glycosylase (UNG) and the MMR factor MSH2 are not able to undergo CSR.[63] These repair pathways, which normally repair mismatches and small lesions in the DNA, have been "reprogrammed" by the immune system to create diversity. First, UNG removes the U to form an abasic site, which is recognized by the endonuclease APE1, which produces a ss break (SSB). If two SSBs are located close to each other on opposite strands, these could thus form a DSB, which could either be blunt or contain overhangs. The MSH2/MSH6 heterodimer could also recognize the U/G mismatches and thereafter recruit the MLH1/PMS2 complex, followed by EXO1 that binds to a nearby nick.[64] EXO1, which can perform excision in both 3' and 5' direction, could then excise DNA until it reaches a nick on the opposite strand, which may result in a DSB.

A prerequisite for this model is that AID binds to both DNA strands. The binding to the template strand, with less exposed ssDNA compared to the non-template strand, seems to be

facilitated by the RNA-exosome,[65] a complex that processes/degrades RNA, possibly by removing RNA from the template strand.

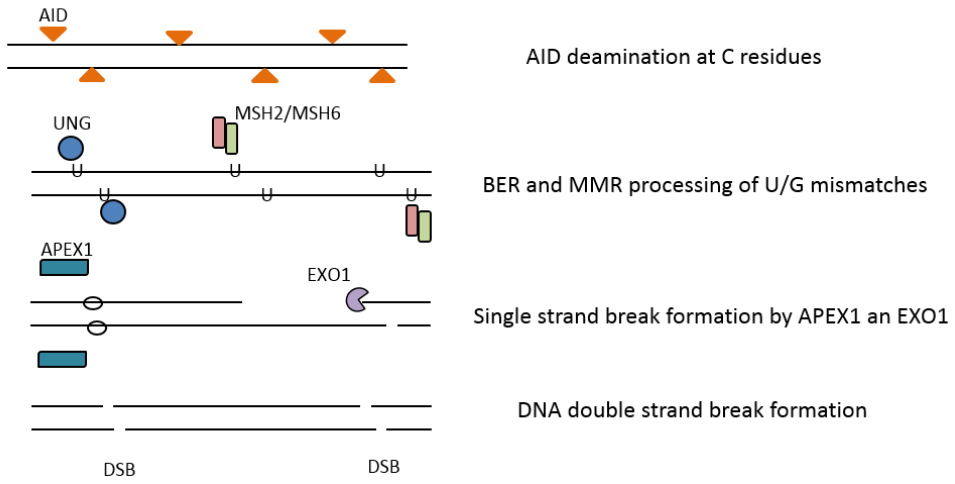


Figure 4. ***DNA double strand break formation through the actions of AID, base excision repair and mismatch repair proteins.*** AID initiates the process by converting cytosines into uracils. These mismatches are subsequently recognized by BER and MMR proteins, which form single stranded breaks (SSBs). Two SSBs that are close to each other on opposite strands may form a double strand break.

1.3.2 Sensing by the DNA damage response

DSB are immediately sensed by DDR factors that coordinate the cellular reaction to the break, such as checkpoint control and DNA repair[66,67]. In addition, several post translational modifications, including phosphorylation, acetylations, methylations and ubiquitinations are induced upon DSB formation[68]. The Mre11-Rad50-Nbs1 (MRN) complex rapidly binds at the vicinity of the breaks and activates P13-K kinases, including ATM and DNA-PKcs, which phosphorylates histone H2AX into γ-H2AX[69]. This modification is recognized by MDC1, which is recruited to the break and attracts the ubiquitin E3 ligases RNF8 and RNF168, which perform ubiquitinations that facilitate the recruitment of 53BP1 and BRCA1. Several DDR factors are important for CSR.

1.3.2.1 ATM

ATM is a Ser/Thr kinase that activates several DDR factors upon DSB formation.[70] ATM-deficiency in humans cause ataxia telangiectasia (A-T, described in section 1.6.3). These patients suffer from immunodeficiency and often present with low IgG and/or IgA levels, which may indicate a CSR defect.[71] In addition, ATM-deficient mice are immunodeficient and show impaired CSR.[72,73] A role for ATM in the repair of DSBs during CSR has also been revealed by the observations of increased MH usage at Sμ-Sγ1 junctions in mice[72] and at Sμ-Sα and Sμ-Sγ junctions from A-T patients.[74] As ATM is involved in the phosphorylation/recruitment/activation of numerous DNA repair proteins, including γ-H2AX, 53BP1 and Nbs1, ATM is likely to affect CSR through several mechanisms.

1.3.2.2 The MRN complex

The MRN complex consists of the Mre11, Rad50 and Nbs1 proteins and Mre11-deficient patients show a reduced CSR efficiency.[75] Furthermore, the Sμ-Sα junctions from Nbs1-deficient patients show increased MH usage, although not to the same level as those derived from cells from A-T-patients, indicating an involvement of this complex in CSR.[75]

1.3.2.3 H2AX, MDC1, RNF8 and RNF168

H2AX is a variant of histone H2 and is phosphorylated after DSB formation in the chromatin surrounding the break. It initiates the recruitment of several other DDR factors, including MDC1, RNF8, RNF168, 53BP1 and BRCA1. Both H2AX-[76] and MDC1[77]-deficient mouse cells show mild CSR defects and an accumulation of IgH locus breaks.[78] RNF8-[79] and RNF168[80]-deficiency also cause CSR defects in mice.[81] Furthermore, two RNF168-deficient patients have been described to date and both presented with low levels of certain Ig classes.[82,83] Moreover, in Paper III we show that the Sμ-Sα junctions from one of the RNF168-deficient patients exhibited increased MH usage[84] and a similar pattern at the Sμ-Sα junctions from the second patient has also been described.[83]

1.3.2.4 53BP1 and RIF1

CSR levels in 53BP1-deficient mice are only 2-10% of those in wild type mice.[85] Thus, deficiency in 53BP1 confers the most severe CSR defect compared to all DDR factors. Furthermore, 53BP1-deficient mice show increased numbers of IgH locus breaks[78]. It is still not fully understood why 53BP1 has such a great impact on CSR. However, two central functions of 53BP1 in CSR have been proposed: in promoting synapsis between distant S regions[86] and preventing end resection.[87] The importance of 53BP1 and its effector molecule RIF1 in preventing resection, which seems to favor the usage c-NHEJ instead of HR, has been elucidated in several papers in recent years.[88-91] The prevention of resection could also steer the repair during CSR to c-NHEJ, whereas inhibit A-EJ pathways.[87]

1.3.2.5 BRCA1

The gene encoding BRCA1 (Breast cancer, type 1 susceptibility protein) was discovered in 1994 as a breast and ovarian cancer susceptibility gene.[92] BRCA1 has been implicated in numerous processes involved in maintaining genome integrity, including checkpoint control, chromatin remodeling, transcription and HR.[93-95] The BRCA1 protein can form complexes with various proteins through its different domains. BRCA1 contains an N-terminal RING domain with E3 ubiquitin ligase activity, binding to BARD1, a coiled-coil domain, interacting with PALB2 and BRCA2 and two C-terminal BRCT repeats, which forms the A, B and C-complexes through interaction with Abraxas, BRIP1 and CtIP, respectively.[93]

One of the main functions of BRCA1 seems to be in repair pathway choice, by promoting HR during the S/G2 cell cycle phases.[88-91] Although BRCA1 is considerably more prevalent at DSBs during S/G2 phases, when HR is active, it also accumulates at DSBs during the G1 cell cycle, albeit at lower levels.[96] Despite its role in promoting HR, several *in vitro* studies have

suggested an involvement for BRCA1 in the NHEJ pathway,[97-100] although this function has been controversial.[93,101] BRCA1 could possibly affect NHEJ by stabilizing the binding of Ku80 to DSBs[96,102] and by modulating resection.[100] In Paper III, we show that CSR-junctions from BRCA1-deficient B-cells contain several alteration, including increased MH usage and intra-switch deletions, suggesting that BRCA1 affects the repair during CSR.[84]

1.3.2.6 The cohesin complex

The cohesin complex was first discovered as the proteins responsible for sister chromatid cohesion.[103,104] Later, cohesin has been attributed with an expanding number of functions, from regulation of transcription through facilitating long-range chromosomal interactions[105] to chromatin condensation and DNA repair.[106]

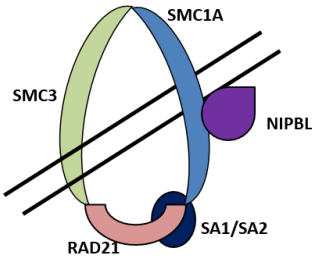


Figure 5. The cohesin complex The cohsin subunits may form a ring structure that could encircle DNA

The multiprotein complex, cohesin, consists of two SMC (structural maintainance of chromosomes) subunits SMC1A and SMC3, a kleisin protein RAD21 and SA1/SA2, which are conserved from yeast to human.[107] These are believed to form a ring-shaped structure that could encircle DNA (Fig. 5). Several cohesin-associated factors have also been found, including the nipped-b-like protein (NIPBL), which loads cohesin to the chromatin.[107]

Already before cohesin was shown to be involved in sister chromatid cohesion, it was observed that RAD21-deficient yeast cells were sensitive to γ-irradiation and exhibited a DNA repair defect.[108] The role of cohesin in DNA repair has mainly been linked with HR, as its ability to hold sister chromatids together is believed to facilitate this process, which is dependent on the close contact between the chromatids.[109]

In Paper I, we show that the Sμ-Sα junctions from NIPBL-deficient patients exhibited increased MH usage, indicating that the repair process during CSR was impaired in these cells.[110] Moreover, a link between cohesin and CSR has been presented in a study published in the same issue as Paper I.[111] The cohesin subunits smc1 and smc3 (mouse counterparts to SMC1A and SMC3) were shown to be recruited to the Sμ region during stimulation of CSR in mouse B-cells.[111] Furthermore, the CSR efficiency was reduced in a CH12 mouse B cell line treated with siRNA for *NIPBL* or genes encoding cohesin subunits.

The involvement of cohesin in transcription seems in part to function through its binding to the CCCTC-binding factor (CTCF) and these proteins can mediate long-range genomic interactions.[112] Cohesin/CTCF can both promote and block transcription, by facilitating promoter/enhancer interactions or by insulating such contacts, respectively.[109] Several CTCF binding sites have been found at the mouse Igh locus and RAD21 have shown a lineage dependent binding to these.[113,114] Moreover, locus contraction was impaired in the absence of CTCF[113] and it has been suggested that cohesin could be involved in this process.[115] In addition, RAD21 has been shown to bind to several TCRα cis-elements, including the Eα

enhancer, and was involved in regulating transcription and H3K4me3 histone modifications, which facilitates RAG recruitment.[116]

1.3.3 Repair by the non-homologous end-joining pathway

Similar to V(D)J recombination, CSR occurs during the G1 cell cycle phase.[117] Furthermore, AID, like the RAG complex, has been shown to steer the repair of the DSBs towards the NHEJ pathway though the interaction with several NHEJ factors to its C-terminal end.[118,119] Deficiency in any of the c-NHEJ proteins seems to confer a CSR defect to various degrees.

1.3.3.1 Ku70/80

The Ku70/80 heterodimer encircles the DNA ends (Fig. 6) and forms a scaffold for subsequent recruitment of c-NHEJ factors. Ku-deficient mouse cells show CSR levels of 20-50% of those in wild type cells.[120] Furthermore, the CSR-junctions from Ku70-deficient mouse cells exhibited increased MH usage, but the repair by direct end-joining was not completely lost.[121]

1.3.3.2 DNA-PKcs

DNA-PKcs is a Ser/Thr kinase and the catalytic subunit in the DNA-PK complex, also containing the Ku70/80 heterodimer.[122] The role of DNA-PKcs in CSR has been unclear with studies showing various effects of DNA-PKcs-deficiency on CSR efficiency.[123-128] Only one of the studies analyzed the repair pattern at CSR junctions and did not find any alterations.[126] However, in Paper IV we show that the CSR-junctions from DNA-PKcs-deficient patients have increased MH usage. Possible functions of DNA-PKcs in CSR could be to activate the Artemis activity,[127] as well as in DNA end synapsis.[129]

1.3.3.3 Artemis

Studies in both mice[127,130] and in humans[131] have shown an involvement of Artemis in CSR. Furthermore, Artemis-deficient mouse B-cells exhibit more AID-dependent IgH locus breaks than control B-cells.[127] Artemis has been proposed to be involved in the processing of DNA ends during CSR by cleaving overhangs.[131] The Sμ-Sα junctions in patients show a complete lack of direct end-joining at Sμ-Sα junctions and an increased usage of long MHs.[131] However, Sμ-Sγ1 junctions are normal both in mouse[130,132] and in humans.[131] Although an increase in $S\gamma_x$-$S\gamma_y$ sequential switching, which could be a characteristic of impaired NHEJ, has been observed in Artemis-deficient human cells.[131]

1.3.3.4 Lig4/XRCC4/XLF

Lig4, XRCC4 and XLF (Cernunnos) are involved in the ligation step of the DSB ends.[35] Whereas Lig4 performs the ligation, XRCC4 is required for its stabilization.[133] The interaction between Lig4 and XLF is slightly weaker, but the addition of XLF to the complex increases the ability to ligate incompatible ends.[35] Furthermore, XLF and XRCC4 have recently been shown to form filaments, which have been suggested to support end

bridging.[134] The complete loss of XRCC4[135] or Lig4[136] is embryonic lethal in mice, which seems to be conferred by the importance of these proteins during neurological development. XRCC4[137,138] or Lig4[121,139] have thus been conditionally deleted in mouse B-cells and these showed reduced CSR efficiency. The repair patterns at CSR-junctions from XRCC4-[137] or Lig4-[121] deficient mouse B-cells were characterized by severely reduced or no direct end-joining and increased usage of longer MHs. XLF-deficient mice are, on the other hand, viable[47] and their B-cells only show only a mild defect in CSR.[47] A shift to MH-mediated repair has also been observed at Sμ-Sα junctions from Lig4-[140] and XLF-[141] deficient patients.

Recently, a new protein with structural similarity to XRCC4, PAXX (PAralog of XRCC4 and XLF), was discovered.[142,143] PAXX was shown to interact with Ku80 and to bind to XRCC4 and XLF during ligation. However, no PAXX-deficient patients or mouse models have been described and it has not yet been investigated whether PAXX is, as XRCC4 and XLF, also involved in CSR.

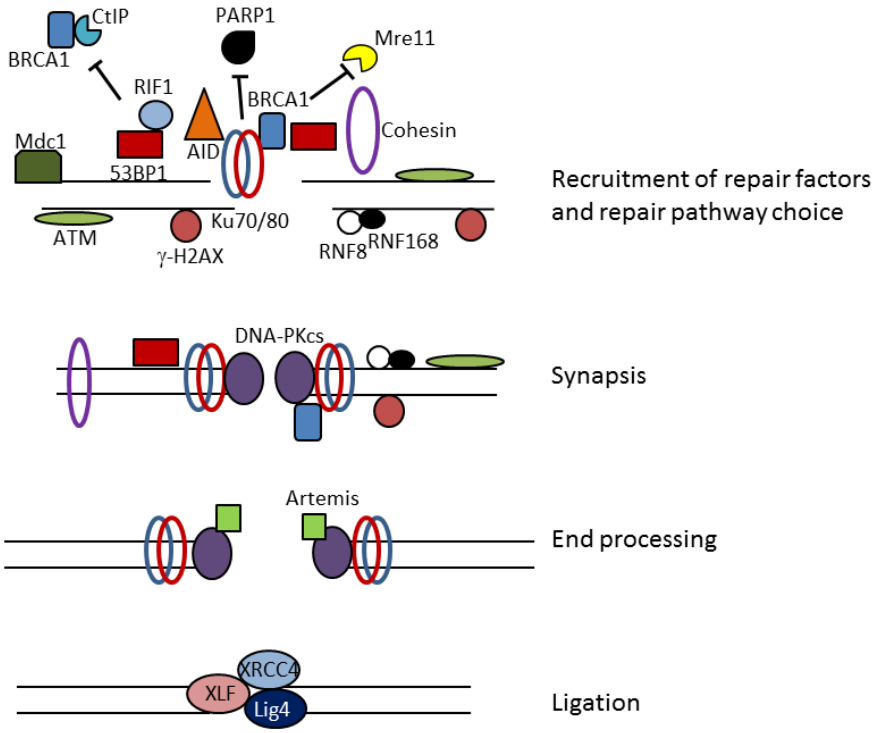


Figure 6: ***Proposed model of repair of AID-induced DSBs during CSR.*** The DSBs are sensed by DDR factors that recruit DNA repair proteins to the break site. The immediate binding of the Ku proteins steers the repair towards c-NHEJ. Thereafter the two DNA ends are synapsed, a process seemingly dependent on DNA-PK complex and DDR factors. If the DNA ends are not blunt, these might be processed by Artemis and DNA-PKcs, before the Lig4/XRCC4/XLF complex can ligate the two DNA ends.

1.3.4 Repair by alternative end-joining

Indications of an A-EJ pathway arose from studies in yeast[144] and mammalian[145] cells, showing that even under c-NHEJ-deficient conditions, linearized plasmids could be repaired, although preferentially through MHs. A-EJ is not clearly defined and it has been debated whether it is a distinct pathway,[37] reliant on a set of "alternative" factors, or whether it is merely an adaption of c-NHEJ, dependent on the missing c-NHEJ protein/s.[35] The terms A-EJ, MH-mediated end-joining[146] and back-up end-joining are frequently intermingled.[37] Although A-EJ often relies on longer MHs, which could possibly reduce the dependence on certain c-NHEJ factors,[35] MHs are not always present.[121]

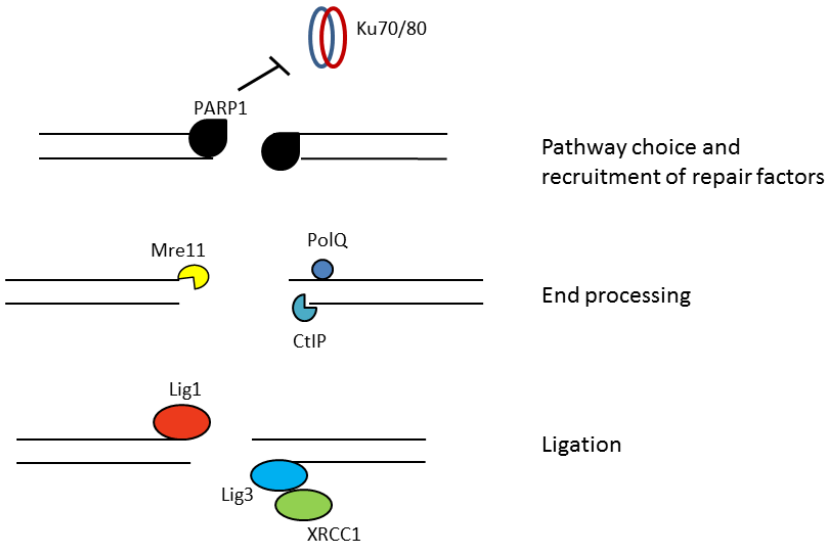


Figure 7. ***Model of alternative end-joining.***

The binding of PARP1 to the DNA ends would inhibit Ku binding and c-NHEJ. Mre11 and CtIP could resect the ends to reveal MHs, which could be recognized by PolQ. The ends might be ligated by Lig1 or Lig3/XRCC1.

It is still not fully elucidated how the choice between HR, c-NHEJ or A-EJ is determined. Nevertheless, the decision between HR and c-NHEJ seems to depend, in part, on the cell cycle phase and the amount of resection around the break. Whereas HR requires extensive resection, long resections could impair the repair by c-NHEJ[88-91]. A-EJ also seems to depend on resection, possibly to reveal sequences for MH-mediated repair. Thus, 53BP1, which has been implicated in repair pathway choice between c-NHEJ and HR, could have a similar function in the decision between c-NHEJ and A-EJ[87].

A number of proteins have been implicated in A-EJ (Fig. 7). Poly(ADP-ribose) polymerase 1 (PARP1) seems to be one of the central players in A-EJ, as it competes with Ku70/80 for binding to the DNA ends, which could channel the repair towards A-EJ or c-NHEJ, respectively[147,148]. In addition, PARP1 has the ability to recruit other factors, such as XRCC1, Lig3 and the MRN complex to the breaks [149]. Furthermore, analysis of CSR junctions in PARP1-deficient mouse cells revealed shorter MHs at these, suggesting a role for PARP1 in MH-mediated repair during CSR[150]. The end resection during A-EJ might be mediated by Mre11 and CtIP[37]. A role for CtIP in A-EJ during CSR was supported by the observation of CSR junctions with decreased MH usage from CtIP-deficient B-cells[151]. Polymerase theta (PolQ) was recently described as a new A-EJ factor[152,153] and could facilitate MH-dependent repair[152,153]. Ligation may be dependent on Lig1 and/or Lig3, as well as XRCC1[149].

1.4 SOMATIC HYPERMUTATION

Although V(D)J recombination creates a highly diversified antibody repertoire it is not sufficient to produce antibodies that bind with high affinity to all possible antigens.[154] Thus, antibodies undergo a second germinal center-dependent diversification process: SHM.[154] This event, similar to CSR, is initiated by AID, which then targets the V regions and the processing of U/G mismatches will instead result in the creation of point mutations or small insertions and deletions. The mutation rate during SHM (~10^{-3}/generation)[154] greatly exceeds the mutation rate in normal cells (~$2.5x10^{-8}$/generation).[155]

Similar as for V(D)J recombination and CSR, transcription is required for SHM. However, the V regions are not as G rich as the S regions and transcription through V regions do not promote R-loop formation.[55] SsDNA needed for AID binding is thus created only by the transcription process itself and SHM is consequently a slower process; SHM requires weeks, whereas CSR occurs withiin a few days.[55]

During SHM, mutations at adenine/thymine (A/T) and G/C positions occur at roughly equal frequencies.[154] SHM is divided into two phases depending on whether the mutations occur at G/C sites (Phase 1) or at A/T sites (Phase 2)[156] (Fig. 8). During phase 1a, replication across the U/G mismatch can create C-T and G-A transitions. Phase 1b is dependent on proteins from the BER pathway. Then UNG can excise the Us and subsequently recruit translesional polymerases, such as Rev1.[154] During the second phase, MMR proteins seem to process the U/G mismatches and recruit error prone polymerases, including polymerase eta, which is known to cause mutations at A/T base pairs. Mice deficient in MSH2 thus have reduced mutations at A/T sites.[63]

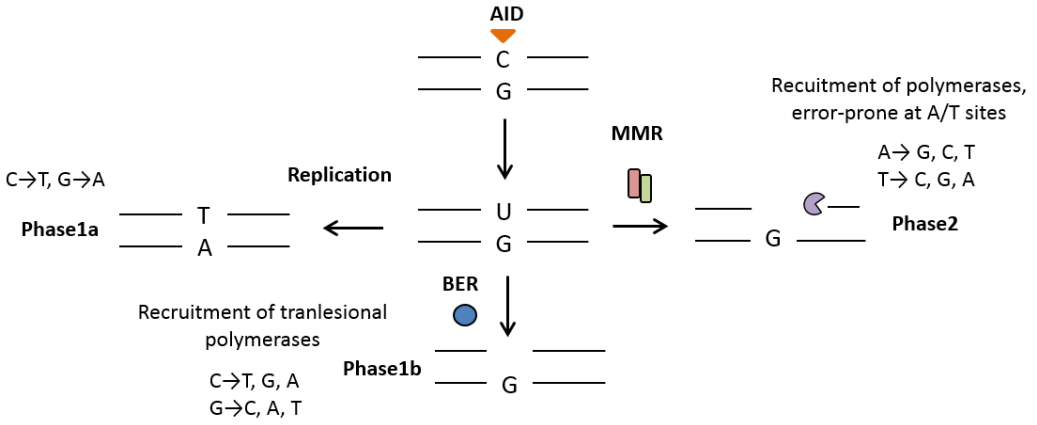


Figure 8. ***Model of somatic hypermutation***
AID induced uracils may be replicated over (Phase 1a), or recognized by the BER (Phase 1b) or MMR (Phase 2) pathways to create mutations at G/C and A/T sites.

1.5 GENE CONVERSION

Gene conversion is a third AID-mediated Ig diversification process, which occurs in chickens, rabbits and other large farm animals. A stretch of 25 pseudo-V genes, without promoters, enhancers and with frequent 5' and 3' truncations, is located upstream of the

functional V genes in the Ig locus of these animals.[157] During gene conversion, pieces of pseudo-V genes are inserted into the V region[158] and, in contrast to CSR, it is dependent on the HR pathway. Consequently, loss of BRCA1 in chicken DT40 cells resulted in reduced gene conversion in these cells.[159]

1.6 PRIMARY IMMUNODEFICIENCY DISORDERS

Primary immunodeficiency disorders (PID) are often caused by monogenic defects in components of the immune system and are associated with high morbidity and mortality if untreated.[160] Over 200 distinct PIDs have been described to date.[161] A subset of these is caused by defective DDR or DNA repair[162] and some of these disorders, related to the thesis, will be discussed below

1.6.1 Severe combined immunodeficiency

Severe combined immunodeficiency (SCID) is one of the most severe forms of PIDs and is in most cases fatal without treatment.[163] It is a rare disease affecting 1:50 000-1:100 000 live births. However, data from newborn screening programs in USA indicate that this might be a conservative estimation and that SCID is more prevalent in certain ethnic groups.[164] SCID patients have defects in the adaptive immune system, which always involve T-cells and in some cases also B- and/or NK-cells. The patients suffer from recurrent infections, which are not cleared without treatment.

SCID has a genetic basis and mutations in various genes involved in B- and T-cell development have been found in SCID patients.[165] Four main mechanisms behind the disease have been described. These include premature cell death of lymphocyte precursor cells, defects in the signaling though the γ-chain dependent cytokine receptors, defective pre-TCR to TCR signaling and V(D)J recombination defects.[165] A subset of the latter group is associated with radiosensitivity (RS) and these patients have mutations in genes encoding NHEJ factors.[166] Until now, RS-SCID has been reported in patients with mutations in *Lig4*, *XLF*, *PRKDC* (encoding DNA-PKcs) and *DCLRE1C* (encoding Artemis).[166] Lig4- and XLF-deficient patients and one case of DNA-PKcs-deficiency[167], in addition, presented with developmental and neurological problems.[166] Recently, several patients with XRCC4-deficiency have been reported.[168-171] However, none of these patients were diagnosed with SCID or even immunodeficiency. Instead they were suffering from developmental and neurological malfunctions.[169] If this is due to a dispensable role for XRCC4 during V(D)J recombination in human cells, or if residual XRCC4 activity in the patient's cells is sufficient to perform V(D)J recombination, has not been determined.

1.6.1.1 DNA-PKcs-deficiency

Two patients with DNA-PKcs-deficiency have been described to date. The first patient carried homozygous mutations in *PRKDC* that seemed mainly to affect its Artemis-activation function.[172,173] This patient suffered from RS-SCID, but did not show any other abnormalities, and resembled the clinical presentations in previously described Artemis-deficient patients.[166] The second patient instead carried compound heterozygous mutations in *PRKDC* of which one seemed to completely inactivate DNA-PKcs, whereas the other caused severely reduced DNA-PKcs protein levels and barely detectable kinase activity.[167] In addition to SCID, this patient was dysmorphic, suffered from severe growth failure and had neurological problems and microcephaly. The neurological malfunction was more severe than those observed in other c-NHEJ-deficient patients.

1.6.2 CSR-deficiencies

CSR-deficiencies, also known as hyper-IgM syndromes, are characterized by elevated or normal IgM levels, whereas absent or low levels of IgG, IgA and IgE and are often accompanied with a defect in SHM.[174] These are rare disorders affecting around 1:100 000 births.[174] CSR-deficiencies can be caused by defects in B-cell activation, such as in patients with mutations in the genes encoding CD40 or CD40 ligand.[175] Other subtypes are due to impaired functions of proteins important for the CSR process itself, including Ig-CSR deficiency 1, which is caused by mutations in *AICDA* (encoding AID) and affects both CSR and SHM.[62] A subclass of AID-deficiency is caused by mutations specifically affecting the C-terminal part of *AICDA*, which is only required for CSR, but not SHM, thus not complemented with a SHM defect. Ig-CSR deficiency 2 is caused by mutations in the gene encoding UNG, which is also important for both CSR and SHM.[174]

In addition, a few patients with defects in DDR or DNA repair proteins and immunodeficiency that could imply a CSR-deficiency have been reported[162]. These include patients deficient in Nbs1[176] or ATM[177] (see section 1.6.3) and the MMR proteins PMS2[178] or MSH6.[179] In addition, a patient with homozygous mutations in *MSH2* presented with childhood malignancy and IgA-deficiency.[180] Furthermore, patients with MSH5-deficiency have been reported with low IgA levels and common variable immunodeficiency (CVID).[181] Two patients with homozygous or compound heterozygous mutations in *RNF168* have also been described.[82,83] The first patient was suffering from Riddle syndrome (radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties).[83,182] The immunodeficiency somewhat resembled a CSR-deficiency disorder as the patient had normal IgM, but low IgG levels and the IgA levels were within the normal range[182]. The second patient had low IgA levels, ataxia and microcephaly.[82] Recently, CSR-deficiency, with a hyper-IgM phenotype, was reported in two patients deficient in INO80.[183] INO80 was shown to co-localize with cohesin at the Ig locus and the authors suggested that the CSR-deficiency could be caused by defective INO80-mediated modulation of cohesin activity during CSR.[183]

1.6.3 Ataxia telangiectasia

A-T is a recessive, autosomal disorder characterized by radiosensitivity, immunodeficiency, cerebellar ataxia, oculocutaneous telangiectasia (dilated blood vessels), growth retardation and increased risk of cancers.[70,71] It is a rare disorder affecting approximately 1:149 000 births in Sweden.[184] A-T is caused by mutations in the *ATM* gene and whereas classical A-T is due to homozygous or compound heterozygous truncating or inactivating mutations, a milder form of the disease, variant A-T, are caused by mutations that leave some ATM activity.[70,71] The immunodeficiency could affect both the humoral and cell-mediated immunity.[185] There are some A-T-patients that present with decreased serum IgA and IgG, but normal IgM, suggesting a CSR-deficiency.[177]

1.7 ADDITIONAL DISORDERS STUDIED IN THESIS

1.7.1 Cornelia de Lange syndrome

Cornelia de Lange syndrome (CdLS) is a developmental disorder affecting multiple systems.[186] Typical clinical features include intellectual disability, gastrointestinal problems, growth retardation, malformations of the upper limbs and characteristic facial features.[186,187] Furthermore, recurrent infections, including otitis media, viral respiratory infections, pneumonia and sinus infections are common[188] and are frequent causes of death in CdLS patients.[189] Although most CdLS patients are not immunodeficient, patients with CVID, hypogammaglobulinemia and specific antibody deficiency have been described.[188]

CdLS is a genetic disease caused by mutations, most often sporadic, in genes encoding members of the cohesin pathway.[187] CdLS is rare and affects about 1:50 000 in the population, although the real number could be higher, since milder cases might escape diagnosis.[190] The majority of patients have heterozygous mutations in the gene encoding the cohesin loader NIPBL. It seems as frameshift/nonsense mutations cause the classical, more severe disorder and missense mutations often result in a milder form, whereas splice site mutations cause a phenotype in between.[186] In addition, CdLS patients with mutations in the genes encoding the cohesin subunits SMC1A, SMC3 and RAD21,[191] as well as PDS5B,[192] which promote cohesin unloading, and HDAC8[193], which deacetylates SMC3, have been reported.[186] In about 30% of CdLS patients, no causative mutation has been found.[186]

The clinical manifestations in CdLS patients are thought to be due to deregulated transcription, particularly during development.[187] The role of cohesin in sister chromatid cohesion is likely to be essential for life, as no null mutations in the cohesin pathway have been reported, hence all patients seem to have some residual cohesin activity.[187] However, other functions of cohesin, such as in DNA repair, might also contribute to the CdLS etiology.

1.7.2 Hereditary breast and ovarian cancer syndrome

Breast cancer is the most common cancer type in women worldwide.[194] About 7% of breast cancers and 14% of ovarian cancer cases are thought to be caused by inherited germline mutations in tumor suppressor genes.[195] The most well-known of these are *BRCA1* and *BRCA2* and individuals with deleterious mutations in these genes run an increased risk of developing hereditary breast and ovarian cancer syndrome.[195] The estimated prevalence of pathogenic *BRCA1* and *BRCA2* mutations is approximately 1:300-800 individuals, although these mutations are more common in certain ethnic groups due to founder effects.[196] *BRCA1* mutation carriers have an approximately 65% risk of developing breast cancer and a 39% risk of ovarian cancer before the age of 70.[197]

Tumors in BRCA carriers are thought to develop according to Knudson's two hit model, with the germline mutation being the first hit, whereas the second allele is inactivated in the tumor.[93] The role of BRCA1 and BRCA2 in HR are believed to be significant for their tumor suppressive functions.[95] However, why breast and ovarian tissues would be more sensitive for HR-defects is far from elucidated.[198] An involvement of BRCA1 in the regulation of estrogen activity has also been suggested to affect breast and ovarian cancer development.[199] No other genes with an equivalent importance for breast carcinogenesis as the BRCA genes have been found. However, several low-penetrance genes, including *ATM*, *CHEK2*, *BRIP1, PALB2, PTEN* and *TP53* of which many belong to the HR pathway, have been reported.[200] Furthermore, *RECQL*, involved in the repair of DSBs, was recently described as a novel breast cancer susceptibility gene.[201]

Knowledge about the mutation status of breast cancer patients could be useful when choosing therapy. In December 2014 the first BRCA1/2-targeted drug, the PARP-inhibitor Olaparib (Lynparza), was approved.[202] PARP-inhibitors exploit the functions of the BRCA proteins in DNA repair, through a mechanism called synthetic lethality. PARP1 is important for the repair of SSBs, whereas BRCA1/2-deficient cells have defects in HR-mediated repair of DSBs. Thus, cells defective in both pathways will be overwhelmed by unrepaired DNA damage, resulting in death of the tumor cells.[203] Furthermore, it has been suggested that PARP inhibition cause error-prone NHEJ-activity in HR-defective cells, which could also contribute to its cytotoxic effect.[204]

Until recently, only mono-allelic mutations were reported in *BRCA1* and as null mutations in *BRCA1* cause embryonic lethality in mice,[205] the occurrence of homozygous mutations was not expected. Nevertheless, two patients with bi-allelic mutations in *BRCA1* have been described.[206,207] Both patients carried one frameshift mutation and one missense mutation in the BRCA1 BRCT domain, thus leaving some residual BRCA1 function. The clinical manifestations of the patients resembled those that are found in patients with Fanconi anemia (FA). FA patients have bi-allelic mutations in genes encoding members of the FA pathway, including the BRCA1-interacting proteins BRCA2, BRIP1 and PALB2.[36] In addition, the

first patient developed ovarian cancer at an age of 28 years, whereas the second patient was diagnosed with breast cancer at 23 years of age.

1.8 LYMPHOMA

Malignant lymphomas are tumors that are derived from the lymphoid cells, either B, T or NK lymphocytes.[208] Lymphomas are traditionally divided into Hodgkin and non-Hodgkin lymphomas (NHLs), of which the latter is a diverse group of malignancies, which include about 75 percent of all lymphoma cases.[208] The incidence of NHL gradually increased between the 1970s and late 1990s and has tripled in the population over 65 years within the last 25 years in the Western world.[208] The reasons behind this rise are not fully understood, but might involve the acquired immunodeficiency syndrome (AIDS) epidemic, better diagnosis and age-related immunodeficiency[208]. About 95 percent of lymphomas are of B-cell origin[209] and it seems to be due to the many DNA alterations that B-cells undergo during CSR and SHM.[209] The most common lymphoma in the Western part of the world is diffused large B-cell lymphoma (DLBCL), which originates from clonal expansions during the germinal center reaction.[210]

1.8.1 The role of lymphocyte receptor diversification processes in lymphoma development

The first indications of a connection between Ig diversification processes and lymphoma development originated more than thirty years ago when translocations involving the Ig locus and the proto-onco-gene *MYC* were observed in lymphomas in humans[211] and in mice.[212] The Ig/MYC translocations often cause an extreme proliferation of B-cells and development of malignancy when the *MYC* gene is put under the control of the strong Ig enhancers (Eμ and/or 3'Cα).[213] Later, similar translocations between the Ig locus and proto-onco genes, such as *BCL2* in follicular lymphoma and *BCL1* in mantle cell lymphoma, have been observed and is a hallmark of many lymphomas.[209] The formation of cancer requires numerous genetic changes, nevertheless, these translocations appear to be important initiation events during the development of some types of lymphomas.[214]

There is a need for DSBs for a translocation to occur and DSBs are intermediates during both V(D)J recombination and CSR. Thus, some of these translocations seem to happen during V(D)J recombination. The RAG enzymes target RSS located in the Ig and TCR loci, however, similar sequences, known as cryptic RSSs, are found elsewhere in the genome. Cryptic RSSs are occasionally bound by RAG, which could induce translocations and such translocations are frequently found in T-cell acute lymphoblastic lymphoma.[214] Furthermore, CCND1/IgH translocations in mantle cell lymphoma and BCL2/IgH translocations in follicular lymphoma have both shown breakpoints close to the RSS sites near the V and J genes.[215]

Translocations may also be induced by AID. Accordingly, formation of c-myc/Ig translocations seems to require AID activity in mouse cells.[216,217] AID appears to have more Ig off-targeting activity than the RAG enzymes and AID binding outside the Ig locus may also create mutations in the genome.[218] Similar as the targeting to the Ig locus, off-target binding seems to be dependent on transcription. According to a study in mice, AID specifically targets promoter-proximal sequences with stalled RNA pol II.[219] Another study found that AID targets super-enhancers and regulatory clusters in the cells from both mice and lymphoma patients.[220] Furthermore, AID off-binding at super-enhancers seems often to occur at the focal regions of overlapping sense and antisense transcription.[221] High levels of AID expression have been observed in Burkitt lymphoma, germinal center B-cell lymphoma and DLBCL, whereas lower levels have been found in subsets of follicular lymphoma and mucosa-associated lymphoid tissue lymphomas.[218]

1.8.2 Alternative end-joining and cancer

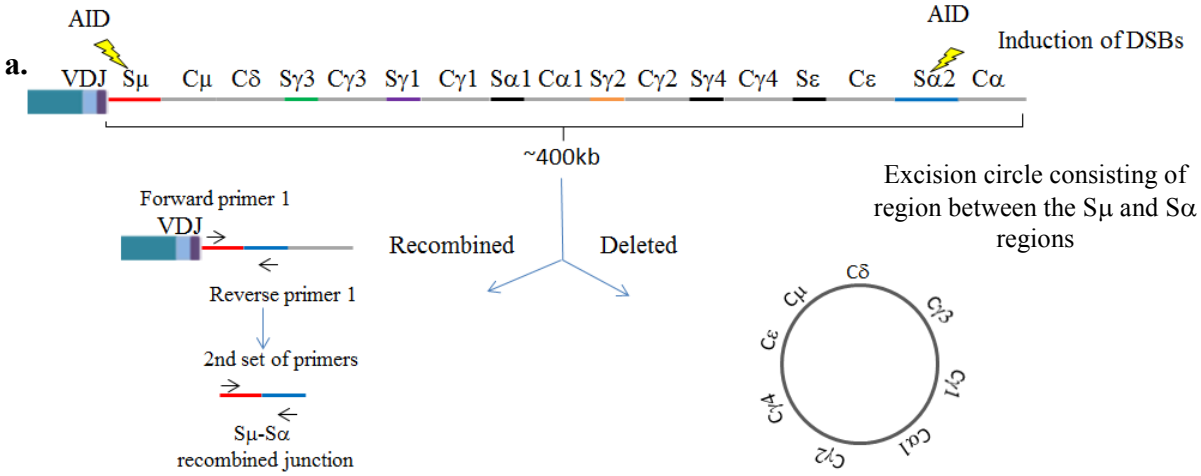
A-EJ is slower and less accurate compared to c-NHEJ and could increase the risk for alterations, such as deletions and insertions, at the recombined junctions.[35,37] Furthermore, A-EJ has been associated with translocations.[120,222,223] Mice double deficient in p53 and certain c-NHEJ factors, such as Ku80, XRCC4, Lig4, DNA-PKcs or Artemis, develop B-cell lymphomas with translocations involving the Ig locus.[224] Moreover, mutations in the c-NHEJ genes have shown to be associated with translocations involving the IgH locus in human B-cell lymphomas.[225]

2 MATERIAL AND METHODS

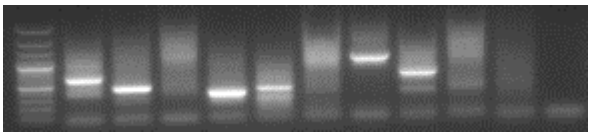
2.1 AMPLIFICATION OF CSR FRAGMENTS AND ANALYSIS OF CSR-JUNCTIONS

In papers I, III and IV, CSR fragments were PCR amplified and sequenced from *in vivo* switched B-cells and the repair pattern at the CSR junctions was subsequently analyzed. The nested PCR assay has been previously described for amplification of Sμ-Sα[226,227] and Sμ-Sγ[140,228] junctions. Genomic DNA, isolated from peripheral blood, was used as a template in a nested PCR reaction with a forward primer binding to the beginning of the Sμ region (Fig. 9a) and a reverse primer to the Sα (binding to either Sα1 or Sα2 due to sequence homology) or Sγ (binding to either Sγ1, Sγ2 or Sγ3) regions for amplification of Sμ-Sα and Sμ-Sγ fragments, respectively. To increase the sensitivity and specificity, a second PCR, with primers located inside the first set of primers, was performed with the PCR1 product as a template. For amplification of Sμ-Sγ fragments, the reverse primers were specific for either Sγ1, Sγ2 or Sγ3 regions. Ten reactions were run in parallel and subsequently visualized on an agarose gel (Fig. 9b). The PCR products could be approximately 100-1500 bp in size, depending on the location of the breaks in the S regions, representing different CSR events. These were then gel purified (Qiagen, Hilden, Germany) cloned in to PGEM-T vector and transformed into DH5α cells and the resulting plasmids were later isolated by Qiaprep mini kit (Qiagen) and sequenced (Macrogen, Soeul, South Korea). A similar strategy for amplification of CSR-junctions from mouse cells were used with primers specific for the mouse Sμ[229], Sα[230] and Sγ (1,2a,2b,3)[231] regions. Genomic DNA from mouse intestine, spleen and blood were used as templates in the nested PCR.

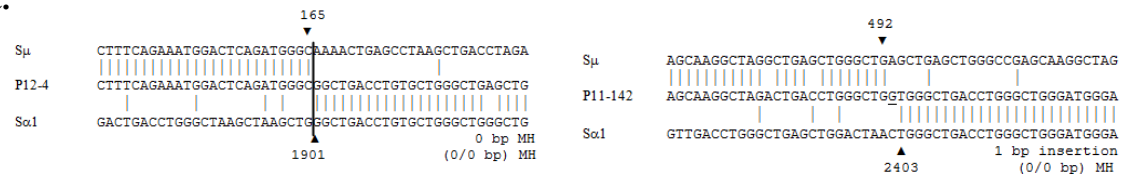
The CSR fragment sequences were subsequently aligned to reference Sμ,[232] Sα[226,233] or Sγ[57,234] sequences for determination of the repair pattern (Fig. 9c). If the two S regions were joined directly, without homology at the putative breakpoints, the repair pattern was nominated direct end-joining. Nucleotide(s) at the junction, not matching either S region, were assigned insertions, whereas a stretch of subsequent nucleotides at the recombined junctions, perfectly matching both S regions, were characterized as MHs. Furthermore, "foot prints" of intra-switch recombination and sequential switching events, as well as inversions can be found at the CSR-junctions (Fig. 9d).



b.

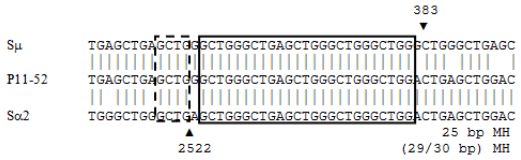


c.



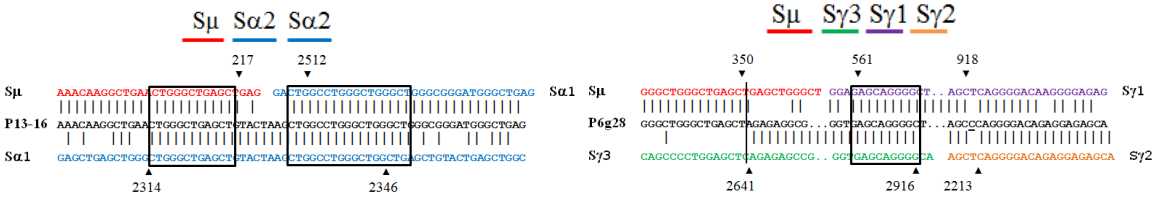
Direct end-joining

Insertion



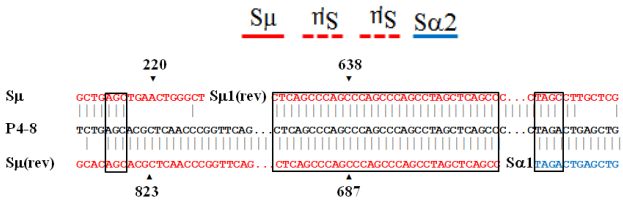
Microhomology-based end-joining

d.



Intra switch-recombination

Sequential switching



Inversions

Figure 9: ***Description of nested PCR-assay for amplification of CSR fragments and subsequent analysis of CSR junctions.***
a) During CSR, AID initiates DSB formation in Sμ and a downstream S region. These are subsequently repaired, resulting in a recombination junction between the two S regions and an excision circle containing the intervening sequence. The forward primer is located in the Sμ region, whereas the reverse primer is located in a downstream S region (here exemplified by Sα), which can amplify the recombined switch regions. b) The products from 10 PCRs run in parallel are subsequently visualized on a gel and bands, representing CSR fragments, are cut out, purified, cloned into bacteria and the plasmids are subsequently sequenced. c) The sequences are aligned with reference S regions and the repair pattern is analyzed. Examples of repair by direct end-joining, insertion and MHs are shown. Direct end-joining is indicated by vertical line, insertion is underlined and MHs are marked by box. The dotted box highlights imperfect repeats, allowing for one mismatch. The position of break points are indicated by arrow heads. d) Examples of CSR-junctions showing signs of intra-switch recombination, sequential switching and inversion events are presented. Neg. cont.: negative control.

2.2 IN VITRO NHEJ- AND V(D)J-ASSAYS

Plasmid based *in vitro* NHEJ- (Papers I and IV) and V(D)J- (Paper II) assays, were performed in cell lines derived from the patient's cells. The assays have been previously described[235] and were modified by us.

For the NHEJ-assay, the pDVG94 plasmid was cut with EcoRV and EcoR47III (Thermo Scientific, Vilnius, Lithuania) to be linearized with blunt ends (Fig. 10). It was then transiently transfected into fibroblast cells using Turbofect (Thermo Scientific). Forty eight hours after transfection, the cells were collected and DNA was isolated using DNeasy blood and tissue kit (Qiagen). The recombined junctions were PCR amplified with FM30 and DAR50 primers[235] and the PCR products were gel purified by QIAquick gel extraction Kit (Qiagen). In the original assay, the relative frequencies of direct end-joining or repair by 6bp MH, facilitated by 6bp identical repeat sequences adjacent to the ends, was assessed by digesting the PCR product with BstXI. Here, to be able to perform a more precise examination of the repair pattern, the PCR products were cloned into PGEM-T vector, transformed into DH5α bacteria and the plasmids were subsequently isolated and sequenced (Macrogen, Soeul, South Korea). The repair pattern was analyzed by aligning the sequences with reference sequences. After sequencing the recombined NHEJ-junctions, other types of repair patterns than the previously described were revealed. These involved deletions, either associated with MHs (deletion + MH) or not (deletions only) (Fig. 11).

For the V(D)J-assay, the circular pDVG93 plasmid (containing RSS sequences), as well as RAG1 and RAG2 plasmids were co-transfected into fibroblast cells and the same procedure as for the NHEJ-assay was followed, with a few exceptions (Fig. 10). For amplification of V(D)J-junctions, a nested PCR with primers NV05F (5'-CTATAGGGGAATTGTGAGCGGATAACDG-3') and DG147 in PCR1 and DG89 and FM30[235] in PCR2 was performed. In the original assay, the PCR product was cut with NotI or NgoMI to assess the proportions of repair by unprocessed joining or 4 bp MH, enabled by 4bp identical repeats at the DNA ends. Similarly to the NHEJ-assay, we have modified the method by cloning and sequencing the amplified recombination junctions. The RAG enzymes could cut the RSS sites and form blunt or staggered ends, depending on the position of the break at the hairpin (see section 1.2.2).The recombined junctions could exhibit similar

repair patterns as the NHEJ-assay (unprocessed, 4bp MH, deletion, deletion + MH) and, additionally, long insertions containing palindromic nucleotides, not observed in the NHEJ-assay, was found in Artemis-deficient cells.

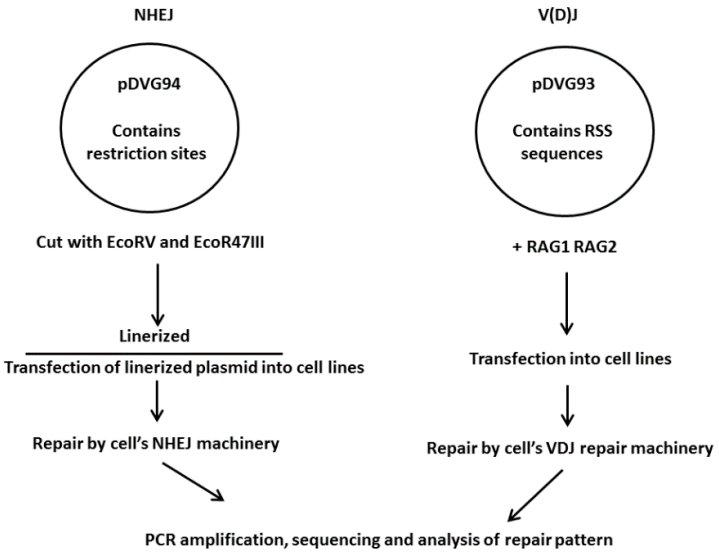


Figure 10: ***Scheme of NHEJ and V(D)J assay.***
Whereas pDVG94 was cut before being transfected into cells for NHEJ-assay, the pDVG93 plasmid, containing RSS sequences, were co-transfected with RAG1 + RAG2 plasmids, to mimic V(D)J recombination.

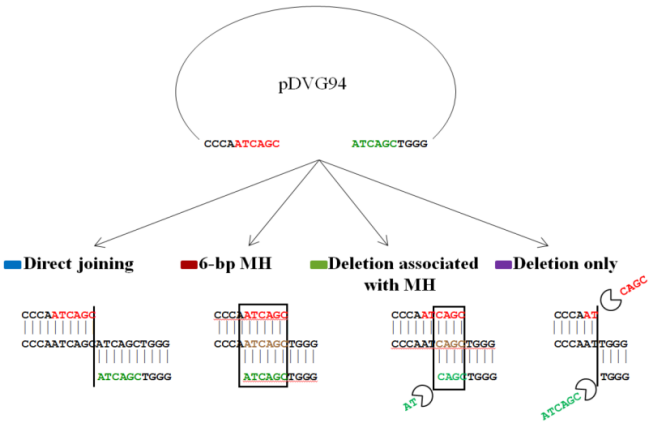


Figure 11. ***Examples of different repair patterns observed at junctions derived from NHEJ-assay***
Similar types of repair patterns were observed after transfection with V(D)J-assay, however, 4bp MHs instead of 6bp MHs were used.

2.3 HIGH THROUGHPUT SEQUENCING OF THE B- AND T-CELL RECEPTOR REPERTOIRES

Sequencing of B-cell receptors by 454

In Paper II, the B- (IgH) and T-cell receptors (TCRβ) were PCR amplified and sequenced by high throughput sequencing platforms.

For amplification of the IgH VDJ regions, a semi-nested PCR containing pools of forward primers, binding to the beginning of the different V gene families, and a reverse primer, binding to the ends of all J gene genes, were used[236] (Fig. 12a). Genomic DNA isolated from peripheral blood was served as a template. The PCR1 product was subsequently used in a second PCR, containing the same primers, but with 10-base multiplex-identifier tails to enable pooling of 12 different samples for sequencing. The high-fidelity Phusion DNA polymerase was used to minimize the occurrence of PCR induced mutations. To increase the specificity of the binding, polymerase was added after a hotstart at 98°C (for 30s, hold at 50°C). In order to produce sufficient DNA for sequencing, repeats of PCR1 (x8) and PCR2 (x2) were performed for each sample. PCR products were gel purified using QIAquick Gel Purification Kit (Qiagen). Samples to be pooled for sequencing were mixed in equal quantities and concentrated using QIAquick PCR Purification Kit (Qiagen) before sequencing on the GS FLX Titanium Sequencer (Agowa GmBH, Germany).

Analysis of gene usage, CDR3 parameters and SHM frequency and pattern

For determining the amino acid sequence at the CDR3 regions, as well as the V-, D- and J gene usage, the IMGT/V-QUEST tool[237,238] (www.imgt.org), allowing for indels, was used. The sequences were organized in an excel sheet for analysis of diversity, V-, D- and J-gene usage and CDR3 properties. Only sequences with unique CDR3s were used for gene usage analysis, to avoid the skew of large, clonal expansions. The sequences were visually inspected for chimeric events, which are artefacts that contain more than one V gene and are created during the PCR reaction.[239] Sequences consisting of another V gene, than that assigned by V-QUEST, at the end, associated with the CDR3 region, were omitted from V gene analysis, as it could not be determined to be unique based on CDR3 sequence.

To study the SHM pattern and frequency, IgH V gene sequences were aligned to the corresponding reference V gene sequence by IMGT/V-QUEST.[237,238] Only unique, unproductive sequences were used for analysis. Chimeric sequences were omitted through the same procedure as for V gene analysis and in cases of chimeric events at the beginning of the sequence, this part was deleted.

Sequencing of T-cell receptors by Illumina Hiseq2000

For TCRβ repertoire analysis, a similar strategy as for IgH V gene amplification was used. However, cDNA was used as template and the TCRβ CDR3s were amplified using an amplicon-rescued multiplex nested PCR method with primers specific for the various V- and C-genes (iRepertoire, Huntsville, USA). PCR1 contained V- and C- gene specific primers, harboring the same end tag sequence that can bind to a communal primer, to allow the specific binding. The PCR1 product was used as template in PCR2, containing the

communal primer that performs the real amplification of the TCRβ V region sequences (Fig. 12b).

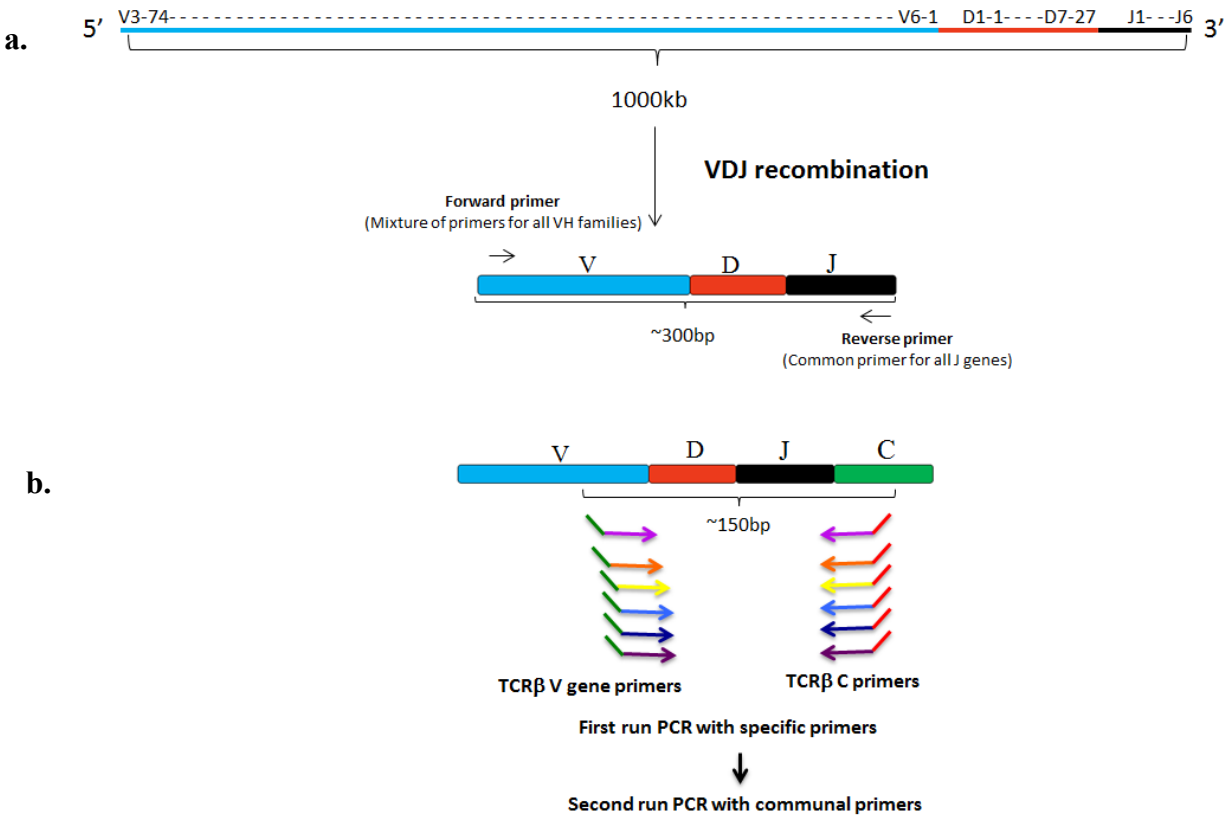


Figure 12: ***PCR strategy for amplification of IgH and TCRβ V regions.***
a) Pools of forward primers, binding to the various V gene families, and a common reverse primer binding to all J genes are used in a semi-nested PCR for amplification of IgH V regions b) TCRβ V regions are amplified by a nested PCR. PCR1 contains primers, with the same tag at the end, specific for the various V and C region genes, whereas PCR2 contains a communal primer that binds to the same tag in all sequences to avoid PCR induced bias.

The PCR2 products were visualized on an agarose gel and purified using QIAquick gel extraction Kit (Qiagen). Equal amounts of DNA with different barcodes were pooled for high throughput sequencing (Illumina HiSeq 2000, iRepertoire). Sequences were aligned to their germline V-, D- and C-genes by the Smith-Waterman algorithm and the V-, D- and J-genes were assigned according to the IMGT/GENE-SB reference directory. Sequences were filtered by SMART (Sequencing error, mosaic sequence, amplification, reference, frequency threshold) filter, which filters out sequencing artefacts, PCR artefacts including chimeric sequences, insertions, deletion and substitutions and CDR3s that present at exceptionally low frequencies were excluded. Analysis of diversity, gene usage and CDR3 characteristics were performed in excel.

3 AIMS

3.1 GENERAL AIMS

The overall aim of this thesis was to elucidate the molecular mechanisms operating during the antibody diversification events V(D)J recombination, CSR and SHM in human cells. These processes are dependent on DDR and DNA repair factors, as well as proteins involved in histone modifications and transcription. By studying antibody diversification in patients with mutations in genes encoding such proteins, we aim to learn more about these processes in human cells, as well as how these defects could affect the patients, particularly the function of their immune system.

3.2 SPECIFIC AIMS

I. The aim of Paper I was to understand the role of the cohesin loader NIPBL in DNA damage response and, in particular, if NIPBL had a function during the NHEJ process. Furthermore, we would like to examine whether NIPBL-deficiency had an impact during CSR in the patient's B-cells.

II. The focus of Paper II was to learn more about the invovlement of cohesin during antibody diversification by studying V(D)J recombination and SHM in the cells from patients with CdLS. We also addressed the question whether the increased frequency of infections in CdLS patients could be due to defects in these processes.

III. The purpose of Paper III was to investigate the role of the tumor suppressor protein BRCA1 and the BRCA1 associated factors BRCA2, BRIP1, CtIP and RNF168 in the NHEJ process during CSR. We were also interested in whether BRCA1 is a tumor supressor for B-cell lymphomas.

IV. The objective of Paper IV was to elucidate the function of DNA-PKcs in the NHEJ process during CSR by studying the only two DNA-PKcs-deficient patients described to date. Since these harbored different defects in DNA-PKcs, an additional aim was to reveal possible kinase -dependent or -independent functions of DNA-PKcs during NHEJ and CSR.

4 RESULTS AND DISCUSSION

4.1 PAPER I.

A regulatory role for the cohesin loader NIPBL in nonhomologous end joining during immunoglobulin class switch recombination

The focus of this paper was to examine the role of NIPBL in DSB repair and particularly its involvement in the NHEJ pathway. To address these questions, cells from CdLS patients, as well as cell lines derived from the patient's cells and yeast strains, were studied. The CdLS patients carried mutations in the gene encoding the cohesin loader NIPBL or the cohesin subunit SMC1A.

4.1.1 Increased DNA damage sensitivity in NIPBL-deficient cells

Sensitivity to DNA damage was examined in B-lymphoblastoid (LCLs) and fibroblast (FB) cell lines derived from 7 CdLS patients. In addition, LCLs and FBs from healthy controls, as well as patients deficient in ATM, XLF or ESCO2, required for establishment of cohesion, were studied. MTS proliferation assay was performed on cells treated with γ-irradiation (g-IR), which induce DSBs, to test their viability after DNA damage. All CdLS cell lines showed reduced survival compared to control cell lines (Fig. 1 in Paper I). Cohesin has previously been implicated in the repair of DSBs by the HR pathway.[106] However, examination of the cell cycle distribution revealed that most (62-88%) CdLS cells were in the G1 cell cycle phase, when HR is not operating. Therefore, we were interested in whether the second major DSB repair pathway, NHEJ, which is pivotal during G1, was impaired in the cells from the patients.

4.1.2 Altered repair pattern at Sμ-Sα junctions from NIPBL-deficient B-cells

As CSR occurs during the G1 cell cycle phase and depends on the c-NHEJ pathway, *in vivo* generated Sμ-Sα junctions were amplified from the B-cells from the CdLS patients. In total, 91 unique Sμ-Sα junctions were analyzed from 7 CdLS patients with mutations in *NIPBL*. The repair by direct end-joining was reduced, whereas the usage of longer MHs (7-9bp) was increased at the junctions from the patients (Table I). A similar shift from the usage of direct end-joining to longer MHs has previously been observed in patients deficient in the NHEJ-factors Lig4 or Artemis (Table I). Thus, these results suggest that the repair by NHEJ during CSR is defective in NIPBL-deficient cells.

4.1.3 Skewed repair pattern at recombined junctions from *in vitro* NHEJ assay

To further study the impact of NIPBL-deficiency on the resolution of DSBs by NHEJ, an *in vitro* NHEJ assay (described in section 2.2) was performed in the CdLS FB cell lines. This assay revealed a shift from the repair by direct end-joining to the usage of longer MHs at the recombined junctions (Table 2 in Paper 1). To ascertain that this phenotype was caused by the NIPBL-deficiency in the patient's cells, the assay was also performed in cells treated with NIPBL siRNA and these cells showed a similarly altered repair pattern. Thus, the NHEJ-pathway appears to be affected in NIPBL-deficient cells, also in a context not involving CSR.

Table I: Summary of Sμ-Sα junction[a] results from all patients studied in this thesis

Study subjects	Perfectly matched short homology						Total no. of S. junctions
	0bp						
	Direct end-joining (%)	Small insertions (%)	1-3bp (%)	4-6bp (%)	7-9bp (%)	≥ 10bp (%)	
BRCA1$^{+/-}$	**21(11)*↓**	40(18)	**45(21)***↓**	32(15)	**38(18)*↑**	**38(18)***↑**	214
BRIP1$^{-/-}$	1(3)	**2(6)*↓**	6(19)	**11(35)***↑**	**10(32)***↑**	1(3)	31
CtIP^{SCKL2}	2(14)	1(7)	3(21)	**5(36)**↑**	1(7)	2(14)	14
BRCA2$^{+/-}$	4(14)	5(17)	10(34)	4(14)	2(7)	4(14)	29
RNF168$^{-/-}$	2(8)	2(8)	**3(12)*↓**	**7(27)*↑**	**8 (31)**↑**	**4(15)*↑**	26
Controls (adult)[b]	41(16)	56(22)	91(36)	29(11)	25(10)	14(5)	256
NIPBL$^{+/-}$	**6(7)↓**	**4(4)↓**	16(18)	14(15)	**32(35)↑**	19(21)	91
SMC1A$^{-/-}$	5(10)	6(12)	13(26)	10(20)	10(20)	7(14)	51
BRIP1$^{-/-}$	8(17)	7(15)	7(15)	11(24)	9(20%)	4(9)	46
BRCA2$^{-/-}$	2(6)	9(29)	4(13)	5(16)	5(16%)	6(19)	31
DNA-PKcs^{P1}	2(5)	6(15)	3(8)	6(15)	**9 (23)*↑**	**13 (33)**↑**	40
DNA-PKcs^{P2}	0(0)	1(7)	1(7)	4(27)	**4 (27)*↑**	5(33)	15
P1+P2	**2(4)*↓**	7(13)	**4(7)*↓**	10(18)	**13(24)*↑**	**18(33)**↑**	55
Artemis$^{-/-c}$	**0(0)**↓**	**6(11)*↓**	10(19)	8(15)	9(17)	**21(39)***↑**	54
Lig4$^{-/-d}$	**0(0)*↓**	**1(3)*↓**	7(23)	4(13)	4(13)	**14(47)***↑**	30
XLF$^{-/-e}$	**2(2)***↓**	16(19)	**7(8)*↓**	12(14)	12(14)	**35(42)***↑**	84
Controls[f] (1-13y)	31(17)	42(23)	36(20)	29(16)	19(10)	26(14)	183

a. Statistical analysis was performed using χ^2 test and significant differences are indicated in bold. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Study subjects shown on top of adult controls in table were compared to adult controls, whereas study subjects below were compared to controls with younger ages (1-13y). NIPBL$^{+/-}$ study group contained individuals with various ages and therefore differences that are significant to both control groups are marked in bold.

b. Previously published Sμ-Sα junctions from adult controls.[84,131]

c. Previously published Sμ-Sα junctions from Artemis-deficient patients.[131]

d. Previously published Sμ-Sα junctions from Lig4-deficient patients.[140]

e. Previously published Sμ-Sα junctions from XLF-deficient patients.[141]

f. Previously published Sμ-Sα junctions from controls with younger ages.[110,131] y:years, bp: base pair

4.1.4 Impaired NHEJ in yeast cells

To test whether the involvement of NIPBL in NHEJ is evolutionary conserved, an NHEJ assay was performed in yeast cells deficient in Scc2, the yeast NIPBL orthologue. As Scc2 is

central for cell survival in yeast, a temperature sensitive *Scc2* allele, *Scc2-4*, was used. Yeast carrying *Scc2-4* were thus arrested in the G1 phase, by treatment with A-factor pheromone, and the temperature was changed to inactivate the *Scc2-4* gene, while a DSB was created in the MATα locus by the activation of an HO endonuclease. This break could solely be repaired by NHEJ, as the regions that would be used for HR-mediated repair were deleted and the DSBs were induced on both alleles. The yeast carrying the *Scc2-4* allele showed poor survival at a level comparable to yeast cells deficient in the c-NHEJ factor Lig4 (Fig. 3 in Paper I). Hence, the function of NIPBL in NHEJ seems to be evolutionary conserved. Discussion on the role of NIPBL/cohesin in NHEJ can be found in section 4.2.6.

4.2 PAPER II

Altered usage of 3' proximal immunoglobulin variable genes in patients with the cohesinopathy Cornelia de Lange syndrome

In Paper I, we showed that NIPBL is involved in the repair process during CSR. In paper II, we have continued the investigation of the role of NIPBL and cohesin in antibody diversification. V(D)J recombination and SHM were studied in 8 CdLS patients with mutations in either *NIPBL* (n=5) or the cohesin subunit *SMC1A* (n=3). Furthermore, 5 A-T patients, who are deficient in ATM, were examined. We were also interested in whether ATM, similar to 53BP1,[240] could be involved in facilitating long range DNA-interactions during V(D)J recombination.

4.2.1 Reduced IgH repertoire diversity in CdLS patients

To study V(D)J recombination during IgH rearrangement in NIPBL- and SMC1A-deficient CdLS patients, the IgH V regions were PCR amplified from gDNA and sequenced by a high throughput sequencing platform (454). On average, 3863 sequences were obtained from each sample. To obtain an overview of the IgH repertoires, the IgH diversities were examined. Notably, both the IgH diversity (unique/total sequences), as well as the D50-values (the cumulative frequency of sequences that account for half of the repertoire) were reduced in the NIPBL- and SMC1A-deficient patients (Fig. 1 in Paper II). Thus, CdLS patients appear to have a reduced IgH diversity, as well as an IgH repertoire more skewed towards large clonal expansions than healthy controls.

4.2.2 Altered V gene usage in CdLS patients

Studies in mice have shown an involvement of the cohesin complex during regulation of V(D)J recombination at both the IgH[113] and TCRα loci.[116] It has also been suggested that cohesin could facilitate locus contraction, which enables the diverse usage of V genes throughout the AgR loci (Fig. 13). Mouse B cells deficient in factors involved in locus contraction, including Ikaros,[241] Yin Yang 1[242] and Pax5,[243] have shown increased frequencies of 3' proximal, but reduced usage of 3' distal, V genes.

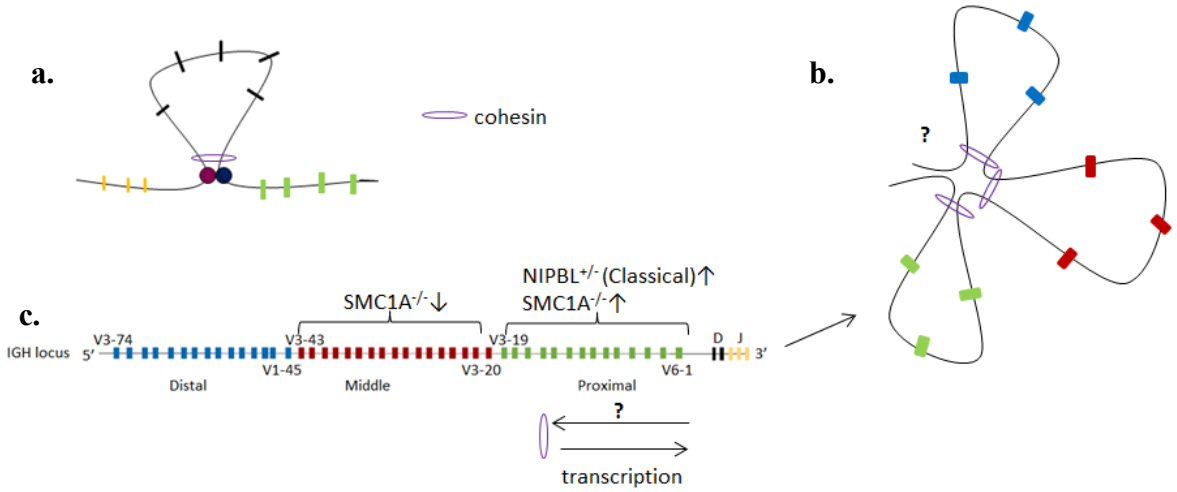


Figure 13: ***Possible roles of cohesin during V(D)J recombination***
a) Cohesin could regulate transcription through mediating promoter-enhancer interactions. b) The IgH locus is compacted, through the formation of loops throughout the locus during V(D)J recombination and cohesin has been suggested to aid this process. c) NIPBL- and SMC1A-deficient B-cells showed an increased usage of the most 3' proximal V genes, which could possibly be caused by altered transcription (germline or antisense) or locus contraction in the cohesin defective cells.

Thus, we were interested in whether the reduced IgH diversities in the patients could be caused by an altered V gene usage. The IgH V regions from the SMC1A-deficient cells and the NIPBL-deficient cells from the CdLS patients with the classical form of the disease, showed a skewed V gene usage, with increased frequencies of the proximally located genes (Fig. 13). A decreased usage of the middle genes was also seen in the SMC1A-deficient cells. This analysis was performed on unproductive sequences, which are not expressed due to frameshifts or premature stop codons, and therefore represent a repertoire that is less influenced by selection. Thus, these results indicate that this alterations are likely caused by changes in the V(D)J recombination process itself, rather than by the selection from antigens. The changed V gene usage could possibly indicate a defect in locus contraction in the cells from CdLS patients. However, the process is not likely to be completely ablated as distally located genes were still used. In addition, an involvement of cohesin in germline or antisense transcription might also be and underlying cause for the changed V gene frequencies. It could possibly be due to impaired insulator activity, resulting in increased transcription of the proximal genes, in CdLS cells[244] (Fig. 13).

4.2.3 Reduced frequency of mutations in the IgH V regions in CdLS patients

Subsequently, the SHM pattern and frequency in the IgH V regions from the B-cells of the CdLS patients were examined. The percentage of mutations was markedly lower at the V regions from CdLS patients compared to controls (Table II in Paper II). In addition, the substitution patterns at the V regions were analyzed. However, apart from a reduction in A-C mutations in the SMC1A-deficient group (Fig. 3 in Paper II), the mutation pattern was largely normal in the V regions from CdLS patients. Transcription levels have shown a correlation with SHM efficiency.[245] One possibility could thus be that the role of cohesin in controlling transcription extends to the regulation of transcription during SHM. It would thus be interesting to study further whether transcription is decreased during SHM in CdLS patient's cells. Moreover, another potential function of cohesin in SHM could be in inducing chromosomal conformational changes, as these appear to aid in opening up the locus in order to enable AID binding.[246,247]

4.2.4 The TCRβ repertoire in CdLS and A-T patients

The TCRβ repertoires were then analyzed in 3 NIPBL-deficient CdLS patients, as well as in 5 A-T patients. The TCRβ V regions were amplified and sequenced by a similar method as the IgH sequences. However, cDNA was used as template and the amplified V regions were sequenced by the Illuminia HiSeq 2000 sequencing platform. On average, 6 220 839 sequences from each sample were obtained by this method. The TCRβ diversities were slightly reduced in the CdLS patients and this trend was even stronger in the A-T patients (Fig. 1, Paper II).

The TCRβ V gene usage was analyzed, but seemed to be less affected in the NIPBL-deficient cells, compared to the IgH V gene frequencies. Nevertheless, a slight trend towards reduced usage of proximal, whereas increased usage of distal, V genes was instead observed (Fig. 4 in Paper II). The TCRβ locus is considerably smaller in size compared to the IgH locus and it is possible that V(D)J recombination is differently regulated at the two loci. Furthermore, only mild CdLS patients were included in the TCRβ V gene analysis and the classical CdLS patients might have shown larger alterations. Thus, the TCRβ V gene usage should be studied in more CdlS patients to determine its role in the process.

The A-T patients showed altered frequencies of several V genes, as well as a similar tendency, as the NIPBL-deficient cells, towards a decreased proximal, but increased distal V gene usage (Fig. 4 in Paper II). A more marked change, however, was observed in the J gene usage. The V gene proximal J1 genes were underused, compared to controls, whereas the frequency of J2 distal genes was increased in the ATM-deficient cells (Fig. 5 in Paper II). A similar shift between the usage of the J1 and J2 clusters have been observed in mouse cells deficient in certain cis regulatory elements, including the CTCF binding sites between the D1J1 and D2J2 regions,[248] as well as the PDβ1 promoter.[249] Thus, besides its function in

stabilizing the DNA ends during DSB repair,[42] ATM might also have a regulatory function, affecting the accessibility of different TCRβ locus regions, during V(D)J recombination.

4.2.5 Normal repair of coding joints in CdLS patients

As we had previously shown that NIPBL affects the NHEJ repair during CSR, we were also interested in whether NIPBL is involved in the NHEJ-mediated repair of coding joints. Therefore, an *in vitro* V(D)J recombination assay (described in section 2.2) was performed in CdLS derived FB lines. Cells deficient in the c-NHEJ factors XLF or Artemis, known to be required for repair of coding junctions in human cells, were also studied. The repair pattern at the junctions from the NIPBL-deficient cells was largely normal, although a decreased frequency of 4bp MH was observed. In addition, no alterations were found at the *in vivo* coding junctions, suggesting that NIPBL does not largely affect NHEJ repair during V(D)J recombination.

4.2.6 Paper I and II. What could be the mechanism behind the involvement of NIPBL in NHEJ?

Cohesin had previously only been implicated in the repair through the HR pathway. Nevertheless, the results presented in Paper I showed that NIPBL-deficient cells had increased sensitivity to DNA damage and impaired capability to repair these by the NHEJ pathway. No cohesin-independent function of NIPBL has been described and the defect is thus likely due to impaired cohesin loading.

The nearly normal repair pattern observed at the coding junctions from NIPBL-deficient cells might seem contradictory to the role of NIPBL in NHEJ during CSR. However, many DDR and DNA repair factors appear to have various impact on CSR and V(D)J recombination.[43] The RAG proteins are associated with both the coding and signal ends after the cleavage step and seem to facilitate the recombination of the DNA ends.[33] AID probably does not have the same protecting effect on the DNA ends as the RAG complex. Repair of coding and signal ends might thus be less dependent on DDR factors, which during CSR could have an end-tethering function.[43]

According to a previous study, cohesin could be involved in the recruitment of 53BP1.[250] Furthermore, 53BP1-deficient mice have shown a quite mild V(D)J recombination defect,[85,240] but severely impaired CSR. To test the hypothesis that cohesin affects NHEJ by impairing the recruitment of 53BP1 to DSBs, 53BP1 foci formation was investigated in the NIPBL-deficient LCLs. Notably, 30 min after treatment with γ-IR, the 53BP1 foci formation was significantly reduced in the NIPBL-deficient cells, compared to the control cells (Fig. 4 in Paper I). Ninety minutes after γ-IR, this difference was no longer noticeable. This suggests that NIPBL is required for the early recruitment of 53BP1 to DSBs. Since 53BP1 seems to

promote c-NHEJ, as opposed to A-EJ, the impaired 53BP1 recruitment in NIPBL-deficient cells might thus shift the repair to A-EJ during CSR, as proposed in Fig. 14. Furthermore, NIPBL/cohesin could possibly facilitate the recruitment of other DDR or NHEJ factors during CSR, which could also influence the repair during CSR. In addition, cohesin might have additional functions during CSR, similarly as during V(D)J recombination, in the regulation of transcription and in promoting long-range DNA interactions,[111,115] which could possibly affect AID targeting.

Furthermore, an involvement of NIPBL in the repair of coding ends, through the recruitment of 53BP1, could be masked by functional redundancy with other proteins, as shown previously between 53BP1 and XLF in mouse cells.[49] We have not tested the role of NIPBL during the repair of signal ends. The signal ends, however, appear to be even less dependent on DDR factors, possibly as they are extrachromosomal and seem to have a stronger association with the RAG enzymes compared to the coding ends.[33] As the VDJ-assay used in Paper II mainly assess qualitative changes at the repair pattern, an assay that measures the efficiency of V(D)J recombination should also be performed in the NIPBL-deficient cells.

4.2.7 Paper I and II. Could CdLS be an immunodeficiency disorder?

The results presented in Papers I and II suggest that all three antibody diversification processes are affected in CdLS patients. Most patients deficient in c-NHEJ factors are presented with SCID,[166] as c-NHEJ is central for the repair of DSBs during V(D)J recombination. Somewhat milder immunodeficiencies are observed in patients with defects in certain DDR factors, such as the immunodeficiency in A-T patients.[70] In addition, the two reported RNF168-deficient patients had normal levels of IgM combined with either low IgG or IgA levels[82,83] and patients deficient in the chromatin remodeling factor INO80 presented with a hyper-IgM phenotype.[183] On the contrary, CdLS is not even considered an immunodeficiency disorder. However, a recent study suggests that CdLS patients suffer from recurrent infections that are sometimes fatal and immunodeficiency has been reported in selected patients.[188] It seemed that the repair of coding joints during V(D)J recombination was not largely altered, whereas the repair during CSR was affected, in NIPBL-deficient cells. If CSR is impaired in the absence of NIPBL, how come not more patients show signs of immunodeficiency? Although both AID and the RAG enzymes steer the repair towards c-NHEJ, V(D)J recombination appears to be more reliant on this pathway and A-EJ is often not operating even in the absence of c-NHEJ factors.[39] However, the A-EJ pathway has been shown to be quite robust during CSR[120] and could probably compensate defects in c-NHEJ to some extent. Furthermore, as long as CSR is not fully eliminated, the B-cells that have been able to switch could go through clonal expansions and produce Igs, which could possibly mask a partial CSR-deficiency.

The study of IgH V regions in CdLS patients revealed a lower IgH V region diversity, a skewed V gene usage and decreased frequency of mutations in the IgH V regions. These

more subtle defects in antibody diversification, and possibly in TCR development, might also result in impaired immune responses to infections in the patients. CdLS is a disease with variable symptoms and depending on mutation status (gene and severity), as well as environmental triggers, more severe immune defects might be observed in certain patients. Hence, it should be considered that the increased infections detected in CdLS patients might not only be caused by anatomical abnormalities, but also by inborn immune defects.

4.3 PAPER III

Aberrant recombination and repair during immunoglobulin class switch recombination in human BRCA1-deficient B-cells

In Paper III, we wanted to investigate the role of BRCA1 and BRCA1-interacting/associated proteins in NHEJ. Previous studies on the involvement of BRCA1 in NHEJ *in vitro*, have yielded conflicting results.[93] The analysis of CSR junctions from BRCA1-deficient individuals' B-cells could thus serve as a suitable model for determining whether the NHEJ pathway is affected *in vivo* in a physiological context. Fifteen patients with deleterious mutations throughout the *BRCA1* gene, as well as patients deficient in the BRCA1 associated proteins BRCA2, BRIP1, CtIP and RNF168 were thus studied.

4.3.1 Longer MHs at Sμ-Sα junctions from BRCA1-, BRIP1-, CtIP- and RNF168 defective B-cells

In total, 227 Sμ-Sα junctions were characterized from BRCA1-deficient individuals. Analysis of the repair pattern at these, revealed a change from the usage of direct end-joining and short MHs (1-3bp) to the usage of longer MHs (>6bp) (Table I). Thus, it suggests a shift from c-NHEJ to A-EJ in BRCA1-deficient cells. Furthermore, the study subjects were grouped depending on whether their mutations had previously shown a specific effect on the BRCA1 protein (Table S2 in Paper III). As the CSR junctions from individuals with mutations affecting the BRCA1 BRCT domain showed alterations, we also included patients with defects in the BRCA1 BRCT interacting proteins BRIP1 and CtIP. The Sμ-Sα junctions from these patients had increased usage of longer MHs, although not as long as observed at those from BRCA1-deficient cells (Table I). CtIP has been implicated in A-EJ and seems to promote resection.[151] It was therefore somewhat unexpected that the junctions from CtIP-compromised cells showed longer MHs. However, the patients in the study carried mutations resulting in the expression of both normal level of wild type protein and a C-terminal truncated variant.[251] This mutation might thus not ablate resection completely, but rather affect the resection capacity favoring the slightly longer (4-6bp) MHs. The other BRCA1 BRCT formation complexes have also been implicated in resection, particularly through

inhibiting the process.[100,252,253] In summary, these results suggest that defects in BRCA1, as well as in BRCA1-interacting proteins, affect the repair by NHEJ during CSR. A potential function of BRCA1 in NHEJ could be to inhibit resection, which would favor c-NHEJ instead of A-EJ as proposed in Fig. 14. In addition, its role in stabilizing Ku80 binding[96,102] to DSBs, is also likely to influence CSR.

Several recent studies have pointed out BRCA1 as a central player in promoting HR during the S/G2 phases by inhibition of 53BP1 and thereby NHEJ.[91,254,255] This function of BRCA1 might seem contradictory to its role in promoting c-NHEJ during CSR. However, this could reflect the versatility of BRCA1, as it seems to have many separate functions, depending on its interaction partners.[256] The HR-defect in BRCA1-deficient cells appears to be partly caused by improper 53BP1 binding, which results in inhibition of resection and impaired HR. However, by knocking down of 53BP1 in BRCA1-deficient cells, the HR defect could be rescued.[254,255] Whether the combined deficiency in BRCA1 and 53BP1 could have a similar rescue effect on NHEJ has not been studied. Hence, we decided to examine the CSR-junctions from an RNF168-deficient patient, whose cells have shown defects in the recruitment of both BRCA1 and 53BP1 to DSBs.[82] The repair pattern at the CSR junctions from RNF168-deficient cells showed an even stronger dependence on MHs, compared to the BRCA1-deficient cells (Table I). Thus, the combined deficiency in BRCA1 and 53BP1 does not appear to rescue c-NHEJ, but rather cause a more severe defect in the process (Fig. 14).

4.3.2 Additional alterations at CSR-junctions from cells deficient in BRCA1 or BRCA1 associated factors

The CSR-junctions from BRCA1-deficient cells contained higher frequency of intra-switch deletions (ISDs), compared to controls (Table 2 in Paper III). ISDs occur when two DSBs within a homologous S region are joined, instead of between two heterologous S regions (Fig. 9d). Increased ISDs have previously been observed in models deficient in several DDR or NHEJ proteins.[73,86,120,257] The reasons behind increased ISDs are not fully understood, but ISDs have been suggested to represent impaired long-range joining of distally located S regions[86] and/or uncontrolled resection from existing DSBs.[258] As proposed earlier, BRCA1 might be involved in preventing resection during NHEJ and in its absence more DSBs might be resected, resulting in S region deletions. BRCA1 has also been shown to affect chromatin remodeling[259] and could possibly be involved in the long-range synapsis of S regions.

In addition, unusual insertions containing inverted S region sequences (Table 2 in Paper III, Fig. 9d), which are extremely rare in control cells, were observed at several CSR-junctions from BRCA1-deficient cells. These were also found at elevated frequency in BRCA2-deficient cells and BRCA1 and BRCA2 might therefore be involved in preventing inversions.

Furthermore, the Sμ-Sγ junctions from BRCA1- and RNF168-deficient cells were analyzed. The repair pattern at these was not largely altered (Table S3, Paper III). However, the

frequency of sequential switching, indicated by junctions such as Sμ-Sγ_x-Sγ_y, (Fig. 9d), was increased in the BRCA1-deficient (11% vs. 0% in controls) and RNF168-deficient (21% vs. 0% in controls) cells. We have previously proposed that sequential switching could be another type of A-EJ mediated mechanism operating at Sμ-Sγ junctions.[131] The homology between the Sμ and Sα regions is much greater than between the Sμ and Sγ regions. The latter therefore serve as a less efficient substrate for A-EJ compared to the Sμ and Sα regions.[260] Hence, an Sμ-Sγ junction from an already switched B cell could be used as a template for switching to another IgG subclass, as the Sγ regions share extensive homology and would be suitable substrates for A-EJ.

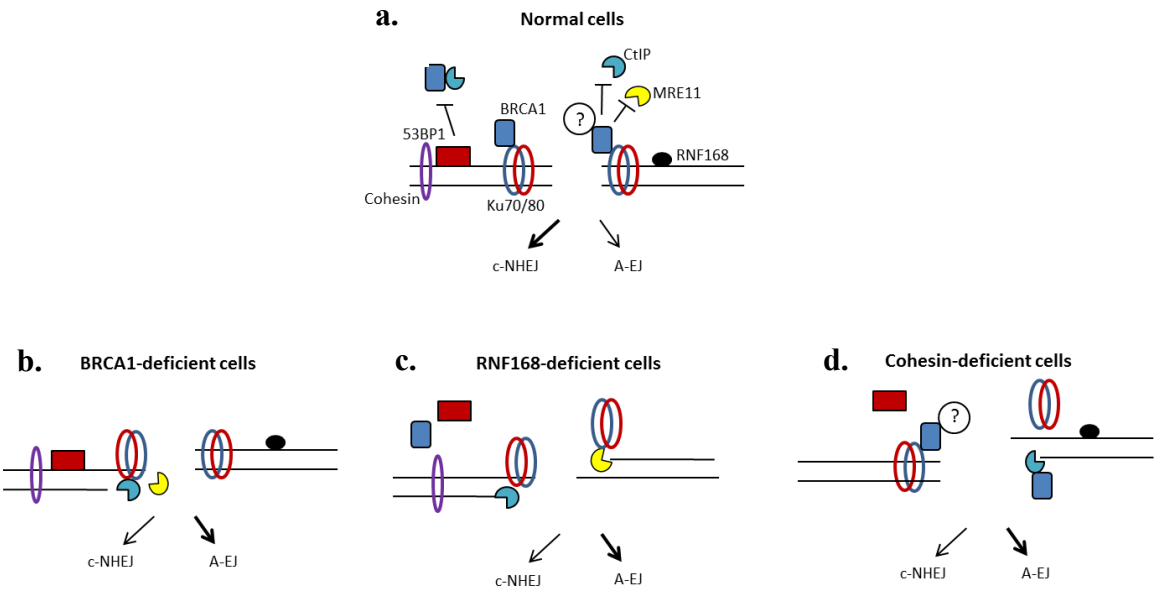


Figure 14. ***Proposed models of contributions of BRCA1, RNF168 and cohesin in NHEJ during CSR.***
a) In normal cells, c-NHEJ is the dominant pathway and BRCA1, possibly in complex with unknown protein(s), and 53BP1 may inhibit resection and promote proper synapsis. In the absence of b) BRCA1 c) RNF168, resulting in impaired recruitment of BRCA1 and 53BP1, or c) cohesin, causing delayed recruitment of 53BP1, resection may be increased and thus shifting the repair towards A-EJ. Furthermore, the synapsis of DNA ends might be destabilized.

4.3.3 Could BRCA1 have tumor suppressor functions during lymphoma development?

BRCA1 is a well-known tumor suppressor gene, a function that seems particularly important in the breast and ovarian tissues. However, other cancers, including melanoma[261] and colorectal cancer in patients below 50,[262] have shown an association with BRCA1-deficiency. Whether defects in BRCA1 could confer increased risk of lymphoma is yet to be resolved. However, a few studies support such a claim. A meta-analysis on studies of carriers of mutations within the BRCA1/2 pathways found a largely increased risk for several hematological cancers, including mantle cell lymphoma and acute myeloid leukemia[263], whereas another study found an increased risk of NHL in patients with certain polymorphisms in *BRCA1* and increased body mass index.[264] Additionally, a novel pathogenic mutation in *BRCA1* was reported in a 56 year old woman who simultaneously suffered from breast cancer and DLBCL and later developed ovarian cancer[265].

Many lymphomas have characteristic gene rearrangements including proto-onco genes and the Ig locus.[209] The many roles of BRCA1 in maintaining genome stability, as well as the aberrantly repaired CSR junctions observed in BRCA1-deficient B-cells, thus make BRCA1 an attractive candidate as a tumor suppressor also for B cell lymphomas. In further support of this, BRCA1/p53-deficient mouse models,[205] as well as mice deficient in the BRCA1-interacting protein RAP80,[266] often develop lymphoma. Consequently, we examined previously published coding genome sequencing data on 31 DLBCLs from our group,[267] as well as unpublished data on additional 22 germinal center related B-cell lymphoma cases for mutations in *BRCA1*. We found a number of somatic and germline mutations or rare SNPs in *BRCA1* in these samples, including a germline, pathogenic frameshift mutation (p.Q1111fs). Notably, the occurrence of rare SNPs or mutations in *BRCA1* (including those with <1% frequency in the ethnically matched population) in our lymphoma cohort seemed to be increased compared to the reported frequency in the general population (Table III). A screening in a larger cohort would, however, be required to determine the contribution of defects in BRCA1 to lymphoma development.

Table III: Comparison of frequencies of SNPs/mutations in *BRCA1* between our lymphoma cohort and the general population

Mutation in *BRCA1*	Frequency in lymphoma cohort[a]	Frequency in population[b]
c.4039A>G	0.019	0.001/0.001
c.3333delA	0.019	Not reported
c.571G>A	0.019	0.001/0.003
c.5347A>C	0.019	Not reported
All mutations combined[c]	0.076	0.004

a. 53 individuals with lymphoma were screened for mutations in *BRCA1*.

b. Allele frequencies in population from 1000 genomes in all ethnicities and in ethnicity matched to patient are shown.

4.4 PAPER IV

DNA-PKcs is required for immunoglobulin class switch recombination in human B-cells both through its kinase- dependent and independent activity

The aim of Paper IV was to determine whether DNA-PKcs is involved in CSR in human cells. Studies on the role of DNA-PKcs in CSR in mouse cells have yielded conflicting results.[123-128] Hitherto, only two patients with DNA-PKcs-deficiency have been described,[167,172] whom showed different defects in DNA-PKcs (Table IV). In order to

understand more about the role of DNA-PKcs in NHEJ, a DNA-PKcs-deficient mouse model was studied. In addition, substrate switching in the Ramos B cell line treated with DNA-PKcs specific (NU7026) inhibitor (DNA-PKcsi) was examined.

4.4.1 Altered repair pattern at CSR-junctions from DNA-PKcs-deficient cells

Altogether, 56 Sμ-Sα junctions were analyzed from the two DNA-PKcs-deficient patients. Similar to the B-cells deficient in the c-NHEJ factors Artemis,[131] Lig4[140] and Cernunnos,[141] the repair by direct end-joining was reduced, whereas the usage of long MHs was increased at the Sμ-Sα junctions from the patient's cells (Table I).

Furthermore, the Sμ-Sγ junctions were analyzed. Whereas no junctions could be isolated from P2, 35 Sμ-Sγ junctions were characterized from P1. The repair pattern at Sμ-Sγ junctions was largely normal (Table S2 in Paper IV). However, the Sμ-Sγ junctions exhibited markedly increased sequential $S\gamma_x$-$S\gamma_y$ switching events (37% vs. 2% in controls) (Fig. 9d) (discussed in section 4.3.2).

As previous studies in DNA-PKcs-deficient mouse models have presented discrepant results, we also characterized 34 Sμ-Sα junctions from DNA-PKcs-deficient mice. In agreement with the results on human cells, these also showed increased repair by long (7-9bp) MHs (38% vs. 13% in controls).

To further investigate the role of the DNA-PKcs kinase activity in NHEJ and CSR, substrate switching in Ramos B-cells cells treated with DNA-PKcsi was analyzed. The Ramos B cell line was stably transfected with the XF-5a plasmid,[268] modified to contain the Sμ and Sα2 or Sγ3 regions (Fig. S2 in Paper IV). After stimulation with CD40 mAb and IL-4, the Ramos cells could undergo substrate switching and the CSR-junctions could be amplified and characterized in the same manner as from *in vivo* switched B-cells. The repair of Sμ-Sα2 junctions from cells without DNA-PKcsi treatment appeared to be shifted towards increased A-EJ in the Ramos system. Nevertheless, after addition of DNA-PKcsi, the Sμ-Sα2 junctions showed an even stronger skew towards A-EJ, implicating that residual c-NHEJ activity in the Ramos B-cells was further repressed in the absence of DNA-PKcs kinase activity (Table V in Paper IV).

Moreover, the Sμ-Sγ3 junctions were studied and these also showed increased MH usage, indicating a shift to A-EJ in the Ramos system. Nevertheless, although the repair by direct end-joining was reduced, it was not fully lost as observed at the Sμ-Sα2 junctions. When adding DNA-PKcsi, however, the repair by direct end-joining was completely abolished (Table V in Paper IV).

4.4.2 Skewed repair pattern at recombined junctions from DNA-PKcs-deficient cells transfected with linearized plasmid

To clarify the role of DNA-PKcs in the NHEJ pathway, the plasmid based *in vitro* assay (described in section 2.2) was performed in FB cell lines derived from the patients. The recombined junctions, from P1 and P2 cells, exhibited a shift to the usage of A-EJ (reduced direct end-joining and increased MH usage). Furthermore, a repair pattern characteristic of deletions associated with MHs was observed at increased frequencies in the cells from P2, but not from P1. Thus, it seems as the cells from P2 harbor additional or more severe defects during NHEJ.

Table IV: Overview of DNA-PKcs-deficient models studied in Paper IV

DNA-PKcs-deficient model	Presence of protein	Kinase activity	Artemis-activation	Effect on Sμ-Sα junctions	Effect on Sμ-Sγ junctions	Effect on plasmid based NHEJ-assay
P1	Yes	Retained	Impaired	MH↑ Direct end-joining↓	Sequential switching↑	Direct end-joining↓ 6bp MH↑
P2	Reduced	Impaired	?	MH↑ No direct end-joining	Could not be amplified	Direct end-joining↓ 6bp MH↑ Deletion+MH↑
DNA-PKcs-deficient mouse	Reduced	Impaired	?	MH↑	Could not be amplified	NA
Ramos + DNA-PKcsi	Yes	Impaired[a]	?	MH↑	No direct end-joining	NA

a. The kinase activity was judged to be impaired based on the effect of addition of the inhibitor on CSR.
P: patient, NA: not analyzed, DNA-PKcsi: DNA-PKcs inhibitor, MH: microhomology.

4.4.3 What could be the kinase-dependent functions of DNA-PKcs during NHEJ and CSR?

The DNA-PKcs-deficient models with impaired kinase activity exhibited additional or more severe effects compared to P1, whose cells had retained DNA-PKcs kinase activity (Table IV). Thus, it seems as DNA-PKcs has additional functions besides Artemis-activation during CSR, which might depend on its kinase activity.

P2 cells showed increased frequency of deletions associated with MHs, which was not observed in the cells from P1, in the *in vitro* NHEJ assay. As deletions would occur by resections around the DSB ends, DNA-PKcs might block the access of the DNA ends to other proteins,[269] thereby protecting them from end-processing before ligation.

The addition of DNA-PKcsi to the Ramos system resulted in a full abolishment in direct end-joining at the Sμ-Sγ junctions. A complete lack in direct end-joining at Sμ-Sγ junctions has previously only been reported in XRCC4-deficient mouse cells.[137] Thus, DNA-PKcs, with inactive kinase activity, might affect the action of XRCC4, possibly through defective phosphorylation of XRCC4 and/or by preventing it from binding to the DNA ends. It seems

as DNA-PKcs auto-phosphorylation is required for the protein to dissociate from the DNA ends.[270] In normal cells, DNA-PKcs protein may shield the ends to prevent resection, however, if the protein does not eventually detach from the ends it might block the access of other proteins, preventing efficient ligation. Notably, DNA-PKcs kinase dead mice have shown a more severe ligation defect, resulting in impaired recombination of signal joints, compared to mice completely deficient in DNA-PKcs protein.[271] An illustration of the possible effects of the different DNA-PKcs-deficient models is shown in Fig. 15.

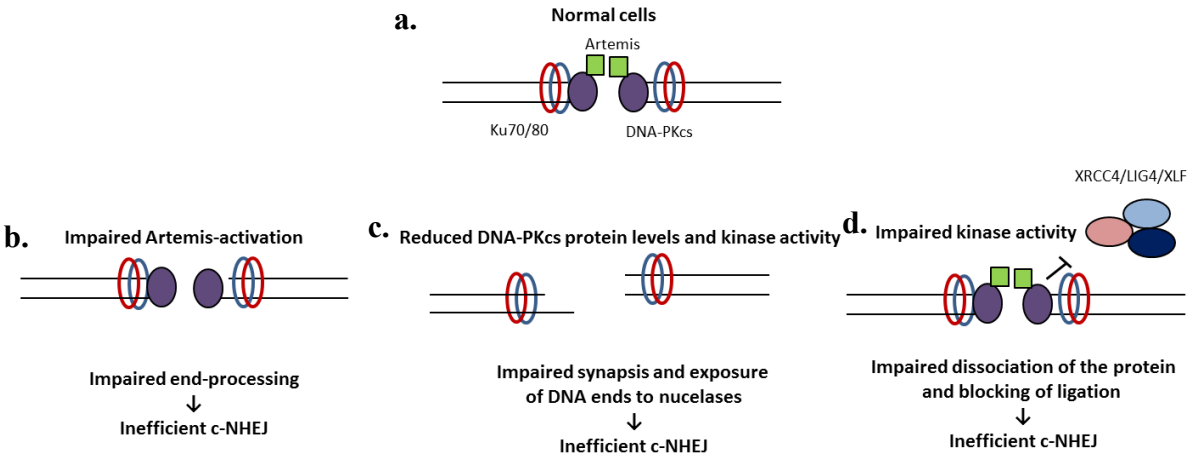


Figure 15. ***Proposed model of the roles of DNA-PKcs in NHEJ during CSR.***
a) In normal cells, DNA-PKcs could activate Artemis, protect the DNA ends before ligation and promote synapsis of the DNA ends. b) In P1, mainly the Artemis-activation function seemed to be affected, which could result in defective end-processing. c) In P2, both protein levels and kinase activity was impaired, which could affect Artemis-activation, end-protection and synapsis. d) The addition of DNA-PK kinase inhibitors to the Ramos system might impair the dissociation of DNA-PKcs from the DNA ends, thus preventing efficient ligation.

4.5 COMPARISON OF CLASS SWITCH RECOMBINATION JUNCTIONS IN HUMANS AND MICE

Papers I, II and III contain the analysis of CSR junctions, which can serve as a useful tool for studying the influence of DNA repair proteins during CSR. Nevertheless, most studies of CSR junctions have been performed in mouse models.[51] Although the human and mouse immune systems share many similarities, the two species separated more than 60 million years ago and many changes have occurred since then.[260] For example, the mouse and human IgH loci differ regarding the number of Cα genes, enhancers and pseudogenes (Fig. 16).

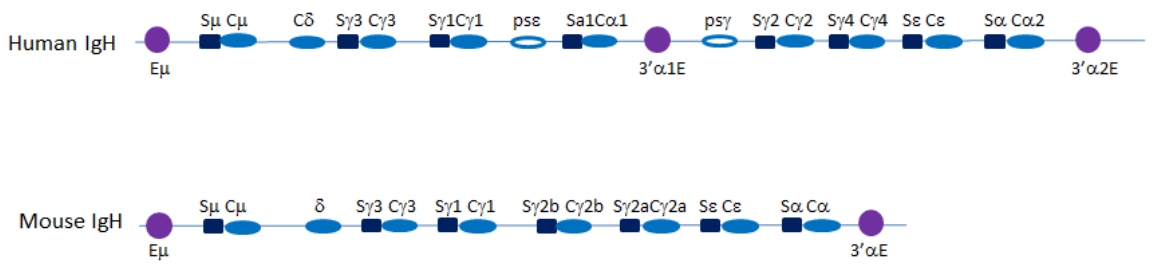


Figure 16. ***The human and mouse IgH loci.***
The human and mouse IgH loci are different, such as the human locus contain two Cα region gene subclasses, two pseudo genes (unfilled rings) and two 3'α1E enhancers.

Since we are mainly studying CSR in human B-cells, it is important to understand whether results from mice are relevant for human conditions or vice versa. There are a few methodical differences between the CSR junction analysis performed in human and mice, which can make the comparison between the two difficult. While studies in human cells have mainly been focused on the Sμ-Sα junctions, most studies performed in mouse cells have analyzed the Sμ-Sγ junctions. As discussed earlier, the deficiency in a repair protein can have very different effects on the repair pattern at Sμ-Sα and Sμ-Sγ junctions. Furthermore, most studies on mice have analyzed CSR junctions from *in vitro* stimulated B-cells, which could potentially alter the repair pattern. The CSR junctions analyzed from humans, on the other hand, have most often been amplified from peripheral blood lymphoid cells, representing the *in vivo* switched B-cells. In order to facilitate the comparison between humans and mice, we have analyzed the Sμ-Sα junctions from *in vivo* switched B-cells from three mouse models, corresponding to three DNA repair disorders that we have studied previously (Table V). The knock-out mice were compared to wild-type mice with the same background strain and with same age, since we have previously shown that the MH usage differ at CSR-junctions from individuals with different ages.[51]

Table V. Patients and mouse models included in comparison

Human disorder	***Mouse model***
Ataxia telangiectasia ($ATM^{-/-}$)	$ATM^{-/-}$
DNA-PKcs-deficiency ($DNA\text{-}PKcs^{-/-}$)	$DNA\text{-}PKcs^{-/-}$
Cornelia de Lange syndrome ($NIPBL^{+/-}$)	$NIPBL^{+/-}$

A comparison of the repair pattern from human control and mouse wild-type cells shows that the frequencies of different lengths of MHs are quite similar in the two species, although the repair by direct end-joining is somewhat higher in the mouse cells (Table VI). In addition, the different mouse strains exhibit some variations, such as the Sμ-Sα junctions from the Cd1 strain have decreased frequency of longer (>7bp) MHs, compared to the B6 and 129/Sv strains. Furthermore, the deficiency in DNA-PKcs or ATM appears to have similar effects at the Sμ-Sα junctions from mouse and human cells, resulting in reduced direct end-joining, whereas increased usage of longer MHs (Table VI). However, this shift is less pronounced in the mouse compared to the human cells. The NIPBL-deficient mice, on the other hand, showed the same amount of direct end-joining as the wild-type mice, although a slight, non-significant, increase in longer (4-6bp) MHs (Table VI).

To conclude, the repair patterns at the CSR junctions are largely similar between healthy human control and mouse wild-type cells. Furthermore, the deficiencies in repair factors, known to influence the repair pattern in human cells (ATM, DNA-PKcs and NIPBL), have a similar effect in mouse cells. However, the alterations appear to be more pronounced in human cells, therefore the results obtained from mouse models could not always be directly extrapolated to a human setting or vice versa. The S region compositions might be responsible for some of the variation between the species. Although the Sμ and Sα regions are the most homologous S regions in both human and mice, the two S regions show even more similarity in humans than in mice.[260] Thus, in the absence of c-NHEJ, the A-EJ would have less homologous substrates to use at the mouse Ig locus, which might result in a slightly weaker shift to long MHs. In addition, the involvement of various DNA repair factors, which could have evolved somewhat differently in the two species, might not be the same. One example is the c-NHEJ factor XLF. Whereas XLF-deficient patients have a CSR defect,[46] XLF-deficient mice show nearly normal CSR levels.[47]

Table VI. Characterization[a] of Sμ-Sα junctions from *in vivo* switched B-cells from A-T, DNA-PKcs-deficient and NIPBL-deficient patients, as well as in corresponding mouse models.

	Perfectly matched homology						
	0bp						
	Direct end-joining (%)	Small insertions (%)	1-3bp (%)	4-6bp (%)	7-9bp (%)	≥10bp (%)	Total no. of S junctions
Study subjects							
A-T[b]	**1(2)*↓**	**1(2)**↓**	15(34)	9(20)	5(11)	**13(30)***↑**	44
Controls (adult)	41(16)	56(22)	91(36)	29(11)	25(10)	14(5)	256
DNA-PKcs$^{-/-}$	**2(4)*↓**	7(13)	**4(7)*↓**	10(18)	**13(24)*↑**	**18(33)**↑**	55
NIPBL$^{+/-}$	**6(7)↓**	**4(4)↓**	16(18)	14(15)	**32(35)↑**	19(21)	91
Controls[c] (1-13 years)	31(17)	42(23)	36(20)	29(16)	19(10)	26(14)	183
Controls[d] (adult)	41(16)	56(22)	91(36)	29(11)	25(10)	14(5)	256
Mouse models[b]					****↑**		
ATM$^{-/-}$ (129/Sv)	2(8)	2(8)	4(16)	7(28)	6(24)	4(16)	25
Wild-type (129/Sv)	14(23)	10(17)	16(27)	9(15)	8(13)	3(5)	60
DNA-PKcs$^{-/-}$ (B6)	4(12)	3(9)	6(18)	4(12)	**13(38)**↑**	4(12)	34
Wild-type (B6)	13(24)	8(15)	18(33)	7(13)	7(13)	1(2)	54
NIPBL$^{+/-}$ (Cd1)	5(25)	2(10)	8(40)	5(25)	0(0)	0(0)	20
Wild-type (Cd1)	5(25)	6(30)	7(35)	2(10)	0(0)	0(0)	20

a. Statistical analysis was performed using χ^2 test and significant differences are indicated in bold. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. A-T and DNA-PKcs$^{-/-}$ patients were compared to controls with lower ages (1-13y). NIPBL$^{+/-}$ study group included individuals with various ages and therefore differences that are significant to both control groups are marked in bold.

b. Previously published Sμ-Sα junctions from A-T patients[74].

c. Previously published Sμ-Sα junctions from controls with younger ages[110,131].

d. Previously published Sμ-Sα junctions from adult controls[84,131].

e. The knock out mice were compared to litter mate wild type mice with same background strain (indicated underneath the mouse model) and age. bp: base pair

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The aim of this thesis was to elucidate the roles of NIPBL, BRCA1 and DNA-PKcs in CSR and NIPBL and SMC1A in V(D)J recombination and SHM in human cells. The main conclusions are:

I. NIPBL-deficient cells from CdLS patients show increased DNA damage. This seems in part to be caused by impaired NHEJ and the function of NIPBL in NHEJ appears to be evolutionary conserved. NIPBL could be involved in NHEJ by facilitating the early recruitment of 53BP1 to DSBs.

II. NIPBL- and SMC1A-deficient CdLS patients show reduced IgH V region diversity. This could in part be caused by a skewed V gene usage in the B-cells from the patients. Furthermore, the IgH V regions from the CdLS patients exhibit a reduced frequency of mutations. These defects in Ig diversification could be an underlying cause of the frequent infections in the patients.

III. BRCA1 affects the repair by NHEJ during CSR. Furthermore, BRCA1-associated factors, including BRIP1, CtIP and RNF168 also seem to influence NHEJ. We suggested a previously unrecognized role of BRCA1 in B-cell lymphomagenesis.

IV. DNA-PKcs is involved in CSR in human B-cells. Furthermore, deficient DNA-PKcs kinase activity seem to confer additional or more severe defects on the process.

This work has shown that NIPBL, BRCA1 and DNA-PKcs are involved in the repair of DSBs during CSR. Still, there are many questions remaining regarding the CSR process.

The discovery of AID about 15 years ago greatly increased the understanding of the molecular mechanisms of CSR. Since then, a myriad of proteins involved in diverse processes, ranging from histone modifications, transcription and the recruitment of AID to BER and MMR pathways, DDR and c-NHEJ, have been associated with CSR.[272] Nevertheless, a coherent picture of the contributions of all the proteins is still missing.

In addition, the A-EJ pathway is yet not fully understood. Whether A-EJ represents one or several pathways, or should not even be designated a pathway, is still open for debate. The factors implicated in A-EJ, such as PARP1, Mre11 and XRCC1, have functions in other repair pathways and do not seem to have developed specifically for A-EJ.[43] A recent screen of A-EJ proteins support the notion that A-EJ employ repair proteins from diverse pathways.[273] Furthermore, the newly recognized A-EJ factor Pol Q[152] was previously known

to be involved in cross-linked repair and BER. As A-EJ is linked to translocations, it would be useful to obtain a deeper understanding of this type of repair and its role in carcinogenesis. Thus, the Ramos B cell line transformed to undergo substrate CSR, which showed a shift to A-EJ and employed in Paper IV, could be a useful system to learn more about this pathway.

Another important question is to further clarify the role of antibody diversification processes in lymphomagenesis. This would include how AID is specifically recruited to the Ig locus. Many of the processes facilitating AID recruitment, such as transcription, occurs elsewhere in the genome and even though AID has Ig off-target activity, its occupancy at the Ig locus exceeds by far any other genomic location.[274] In addition, it would be valuable to learn more about the influence of defects in proteins involved in antibody diversification for lymphoma development. Mutations in DNA repair genes are frequently observed in lymphoma patients[225] and in Paper III several rare SNPs and mutations in *BRCA1* were found in lymphoma patients. An increased knowledge of lymphoma risk genes/mutations could possibly be used for cancer screenings in those with increased susceptibility and thus the earlier discovery of lymphoma. Furthermore, as the newly approved PARP-inhibitor for treatment of breast and ovarian cancer in BRCA1/2 carriers have shown,[202] the knowledge of mutation status and DNA repair pathways could be employed during development of new cancer therapies.

In this thesis the analysis of CSR junctions has been a frequently used tool to study the impact of various DNA repair factors on CSR. Similar as the immune repertoire analysis has moved to the high-throughput sequencing methods in recent years,[275] this might occur for CSR-junctions. High-throughput sequencing of translocations can now be performed[276] and this method has currently been converted for the sequencing of CSR junctions in mice in Fred Alt's lab at Harvard. Adapting this technology for human cells could enable the sequencing of thousands of CSR junctions in a single experiment within a short time period.

A challenge when working with human samples could sometimes be the scarcity of material from rare patients, who might be children or possibly pass away during the study. Then, the novel CRISPR-Cas9 technology,[277] which can make very precise modifications into the genome, could enable the creation of cell lines with patient-specific mutations. The *in vitro* NHEJ and V(D)J assays, used in this thesis, could possibly be performed in such cell lines, making it possible to study the effect of the mutations without sampling the patient.

Furthermore, the expanding number of proteins found to affect antibody diversification offers new candidate genes for patients with PIDs, as the mutation status in most of these patients is still unknown.[278] As recent trials for gene therapy to treat PID patients have shown promising results,[279] the knowledge of the underlying genetic cause of these disorders might become even more useful in the treatment of patients in the future.

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